STUDIES OF THE PHYSIOLOGY OF THE ACANTHOCEPHALAN

MONILIFORMIS DUBIUS (MEYER 1933)

by

S.I. Branch

A thesis submitted for the degree of Doctor of Philosophy in the Australian National University.

March, 1968.

The experimental work reported in this thesis, except for the electron microscopy, was performed entirely by me.

The electron micrograph shown in plate 2.1 was photographed by Dr. W.L. Nicholas.

Stuart I. Branch

(Stuart I. Branch)

25/3/68

FRONTISPIECE

Thirteen week old male (left) and female (right) *M. dubius*.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisors Dr. W.L. Nicholas and Dr. C Bryant for their help, guidance and suggestions. The help of all the academic and technical staff of the Zoology Department, Australian National University, is gratefully acknowledged. Particular thanks are accorded to Mr. J. Dodds for assistance with the liquid scintillation counter and Mr. I. Fox for the photographic work.

I gratefully acknowledge the friendly assistance of Dr. G.M. Watson and his staff of the Department of Radiation Biology at the Australian Atomic Energy Commission Establishment at Lucas Heights, New South Wales.

I thank Dr. D.J. David and his staff of the Division of Plant Industry, C.S.I.R.O., Canberra for advice, instruction and use of their Atomic Absorption Spectrophotometer.

The experimental work was carried out during the tenure of a General Motors Holden's Pty. Limited Post-Graduate Research Fellowship and Special Research and Training Grant No. 66/124 from the Australian Institute of Nuclear Science and Engineering. I would like to express my gratitude to both organisations.

SUMMARY

The osmotic relationships of the acanthocephalan Moniliformis dubius have been examined. The concentration and distribution of the cations Na, K, Ca and Mg have been determined in vivo. The distribution of the Cl ion has been investigated histochemically. Cation movements have been investigated in vitro using weighing experiments to determine the movement of water as indicative of the movement of cations. The movement of Na and K in vitro has also been examined using spectrographic analysis and the radioisotopes Na^{24} and K^{42}.

The nature, time relationships and energetics of water movements have been determined using tritiated water in in vitro cultures.

The characteristics of the uptake of some nutrients from in vitro cultures, in particular the neutral amino acid L - leucine, were investigated using the C^{14} uniformly labelled amino acid. The uptake of this amino acid from media containing other amino acids has also been investigated. The kinetics of these processes are discussed.

Five conclusions are reached from the data:-

1. M. dubius depends upon its external plasma membrane to mediate the exchange of material between itself and its environment.
2. The external plasma membrane has the same characteristics as other cell membranes and is equipped with Na and K pumps.

3. Only the exchangeable Na and K content of M. dubius is osmotically active and causes the organism to be isotonic with the intestine of the rat.

4. M. dubius depends upon its cation pumps for osmoregulation.

5. M. dubius is capable of accumulating amino acids, from in vitro media, in both the pseudocoel and body wall.

SYMBOLS USED THROUGHOUT THE THESIS

The following symbols and abbreviations are used throughout this thesis:

P/coel: Pseudocoel.

P/coel*: Pseudocoel fluids excluding the eggs.

B.W.: Body Wall.

Post.: Posterior.

Ant.: Anterior.

F.: Female.

M.: Male.

Age (weeks): The elapsed time after the rat was infected.

Conc.: Concentration.

μ: The mean.

σ: The standard deviation.

ppm: Parts per million.

Levels of statistical significance are indicated as follows:

NS: Not significant at the 5% level of probability ($p = 0.050$).

* : Significant at the 5% level of probability ($p = 0.050$).

**: Significant at the 1% level of probability ($p = 0.010$).

***: Significant at the 0.1% level of probability ($p = 0.001$).

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CHAPTER 1

GENERAL INTRODUCTION

The members of the Phylum Acanthocephala are bisexual, bilaterally symmetrical, pseudocoelomate parasites of representatives of all terrestrial and aquatic vertebrate groups. The parasites are found attached by their probosces to the walls of the small intestines of their definitive hosts. The sexually mature worm in the gut is the only reproductive stage; thus one fertilised egg gives rise to only one worm in the next generation. Fertilisation is internal. Embryonated eggs produced by the female worm are shed in the faeces of the host and must be eaten by the intermediate host (always an arthropod) before development can continue. The acanthors then escape from their protective shells and penetrate the gut wall of the intermediate host where they develop into juvenile worms in the haemocoel. The definitive host becomes infected when it eats the infected intermediate host. The characteristics of the acanthocephalan life cycle are reviewed by Meyer (1933), Hyman (1951), Petrochenko (1956, 1958), Golvan (1958) and Baer (1961).

Two acanthocephalan parasites were used as experimental animals in the present work. These were Moniliformis dubius (Meyer 1933) and Macracanthorhynchus hirudinaceus (Pallas 1781). Most of the work was carried out on M. dubius, but M. hirudinaceus was employed to obtain comparative data for the cation analysis

described in chapter 3.

The life cycle of M. dubius has been studied by Meyer (1932-33), Burlinghame and Chandler (1941), Yamaguti and Miyata (1942), Moore (1946) and King (1965). Kates (1943) and Meyer (1932-33) have described the life cycle of M. hirudinaceus. The investigations reported here attempt to define some osmotic and nutritional relationships of the worm in its environment. The word "osmotic" is used here as a generic term to describe the distribution of the major cations, Na and K, and of water, both within the worm, and between the worm and its environment in the gut. The nutritional relationships studied concern the uptake of amino acids, in particular leucine, by the worm.

1.1. Historical Survey of the Phylum Acanthocephala.

Golvan (1958) attributes the first published description of an acanthocephalan to Redi (1684), who described the worms found in the gut of eels. Golvan states that the name Acanthocephala was originally derived by Rudolphi (1808-1809) from the German term "Hakenwurmer", used by Zeder (1803), and has since remained the phylum name. It is derived from the Greek words "akantho - spiny" and "kephalo - pertaining to the head".

Golvan (1958) gives the most useful account of the Acanthocephala, presenting a brief history of the early descriptive work, and listing the most important nineteenth and twentieth

century publications, which dealt almost exclusively with anatomy and histology as applied to taxonomy. Early anatomical and histological work on the Acanthocephala was carried out by Kaiser and subsequently Hamann laid the foundations for the modern taxonomy of the group. Meyer (1933) reviewed further taxonomic works published by Travassos and Witenburg. More recent comprehensive taxonomic publications are those of Yamaguti (1935, 1939), Petrochenko (1956, 1958) and Golvan, who published a series of eleven taxonomic papers upon the Acanthocephala in Ann. Parasit. hum. comp. between 1958 and 1962, and a paper with Houin in the same journal in 1963.

Van Cleave is probably the most productive writer on the Acanthocephala as he has published works dealing with many aspects of the group including an important general work on Acanthocephala of North American mammals (Van Cleave (1953)). The author has not seen many of the early works on the Acanthocephala as they are unavailable in Australia and do not have a direct bearing on the subject material of the thesis. The more modern publications which are pertinant to the thesis are reviewed in the appropriate chapters.

The phylogeny of the Acanthocephala has been extensively discussed by Golvan (1958) and will not be reviewed here.

1.2. Taxonomy of the Experimental Animals.

The taxonomy of the two parasites used in this thesis

follows that of Meyer (1933) as modified by Van Cleave (1948). The classifications are:-

Moniliformis dubius.

Phylum	:	Acanthocephala
Class	:	Metacanthocephala. Van Cleave 1948
Order	:	Archiacanthocephala. Meyer 1931
Family	:	Moniliformidae. Van Cleave 1924
Genus	:	Moniliformis. Travassos 1915
Species	:	dubius. Meyer 1933

Macracanthorhynchus hirudinaceus.

Phylum	:	Acanthocephala
Class	:	Metacanthocephala. Van Cleave 1948
Order	:	Archiacanthocephala. Meyer 1931
Family	:	Oligacanthocephala. Meyer 1931
Genus	:	Macracanthorhynchus. Travassos 1917

1.3. General morphology of the Acanthocephala.

The Acanthocephala are cylindrical or flattened worms which may be smooth or show superficial pseudosegmentation. All members of the group lack a gastrointestinal tract at all stages of the life cycle; the body is, in effect, a tubular sac.

The body wall is divided into praesoma, or anterior, and metasoma, or trunk. The division between these two regions of the body is a fibrous partition which penetrates the full depth of the

body wall. The central body cavity is the pseudocoel (see figure 1.1) which extends the full length of the body. The primary holdfast organ of the group, the proboscis, is located at the anterior end of the worm. The proboscis is equipped with hooks, and is eversible in most species. A sac-like organ, the proboscis receptacle, is attached to the body wall anteriorly and receives the invaginated proboscis. The praesoma and metasoma are equipped with a separate system of fluid-filled canals, the lacunar canals. Baer (1961) considers that they serve the function of a circulatory system for absorbed nutrients, but no definitive evidence is at present available. Two tubular structures, the lemnisci, are attached to the body wall anteriorly and hang free in the pseudocoel. These apparently act as a resevoir for the fluids of the praesoma lacunar canal system. The trunk is composed of a syncytium of fibres.

The body wall of M. dubius is covered exteriorly by two plasma membranes overlaid by a fibrous epicuticle, and interiorly by a plasma membrane and basement membrane. Figure 1.2 shows a diagrammatic transverse section of a female M. dubius. Circular and longitudinal muscles are located under the basement membrane and represent the innermost layer of the body wall. The pseudocoel is the central body cavity of both sexes. It contains the reproductive apparatus, and together with the lacunar canal system, may form the hydrostatic skeleton on which the muscles of the body wall operate. The striped layer consists of a series of canals perpendicular to the surface. The radial layer is characterised by radially oriented

FIGURE 1.1.

Diagrammatic longitudinal section of the anterior end of *M. dubius*.

CBM : Circular body muscles.

L : Lemnisci.

LBM : Longitudinal body muscles.

PR : Proboscis receptacle.

PRM : Proboscis retractor muscles.

PRRM : Proboscis receptacle retractor muscles.

PROB : Proboscis.

T : Tegument.

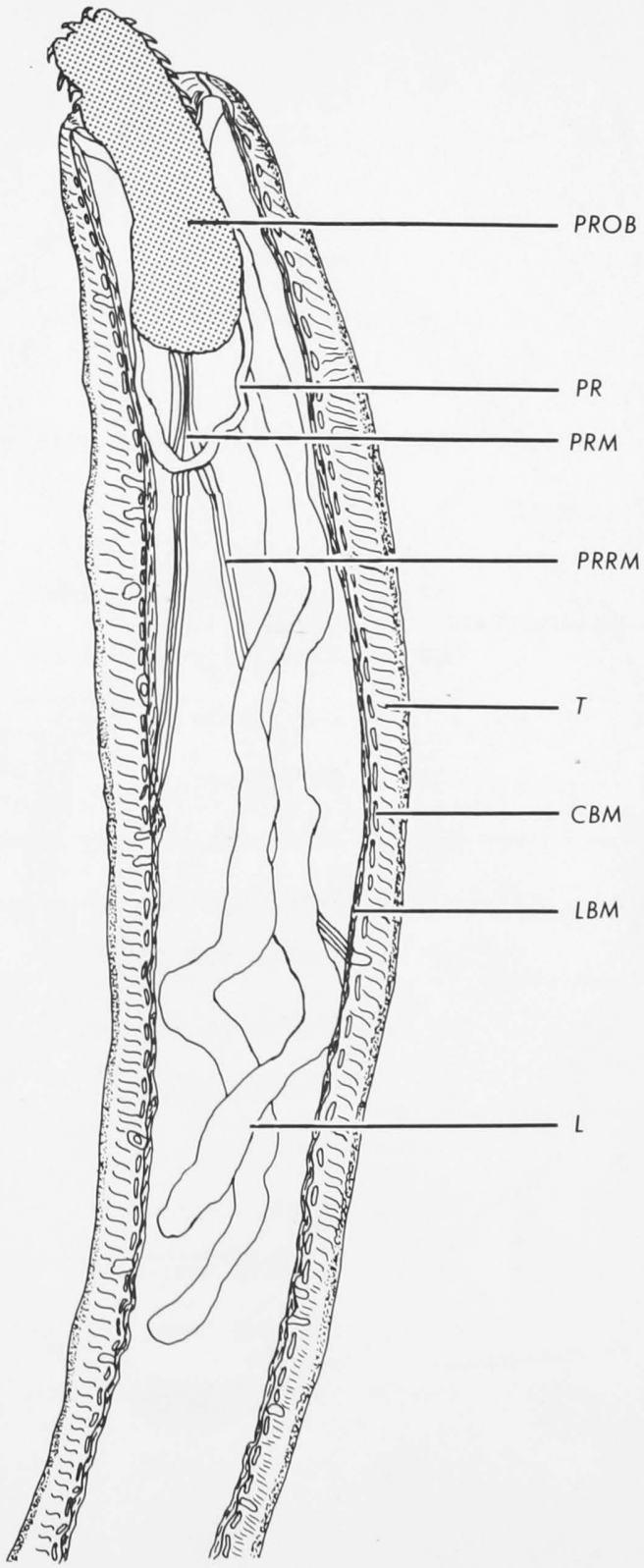

PROB
PR
PRM
PRRM
T
CBM
LBM
L

FIGURE 1.2.

Diagrammatic transverse section of female *M. dubius*.

CBM : Circular body muscles.

DLS : Dorsal ligament sac.

E : Epicuticle.

FL : Felt layer.

LBM : Longitudinal body muscles.

LC : Lacunar canal.

LS : Ligament strand.

N : Body wall nucleus.

OB : Ovarian ball.

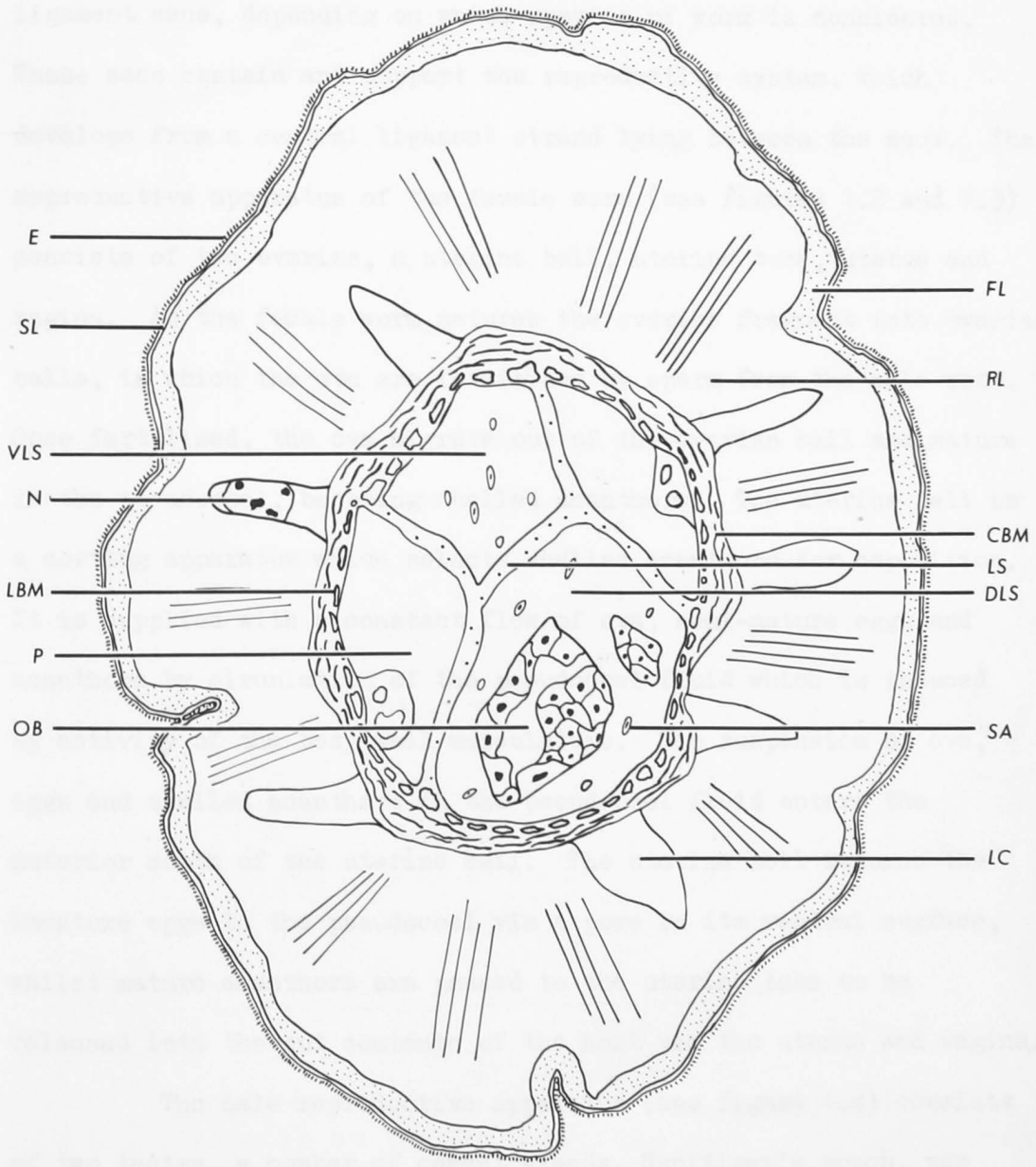
P : Pseudocoel.

RL : Radial layer.

SA : Shelled acanthor.

SL : Striped layer.

VLS : Ventral ligament sac.


E
SL
VLS
N
LBM
P
OB
FL
RL
CBM
LS
DLS
SA
LC

fibres and the felt layer by a feltwork of tegumentary fibres.

The pseudocoel may be divided longitudinally by one or two ligament sacs, depending on which species of worm is considered. These sacs contain and support the reproductive system, which develops from a central ligament strand lying between the sacs. The reproductive apparatus of the female worm (see figures 1.2 and 1.3) consists of two ovaries, a uterine bell, uterine tube, uterus and vagina. As the female worm matures the ovaries fragment into ovarian balls, in which the ova are fertilised by sperm from the male worm. Once fertilised, the ova migrate out of the ovarian ball and mature in the pseudocoel, becoming shelled acanthors. The uterine bell is a sorting apparatus which selects shelled acanthors for deposition. It is supplied with a constant flow of ova, semi-mature eggs and acanthors by circulation of the pseudocoel fluid which is induced by activity of the body wall musculature. The suspension of ova, eggs and shelled acanthors in the pseudocoel fluid enters the anterior mouth of the uterine bell. The uterine bell returns the immature eggs to the pseudocoel via a pore in its ventral surface, whilst mature acanthors are passed to the uterine tube to be released into the gut contents of the host via the uterus and vagina.

The male reproductive apparatus (see figure 1.4) consists of two testes, a number of cement glands, Saeftigen's pouch, vas deferens, penis and an eversible bursa. During copulation, the bursa is everted and grips the posterior end of the female worm. The penis is then inserted into the vagina of the female and sperm are

FIGURE 1.3.

Diagrammatic representation of the reproductive system of female M. dubius (modified after King (1965), and reproduced with the permission of the author).

CBM : Circular body muscles.

DLS : Dorsal ligament sac.

GS : Genital sheath.

LBM : Longitudinal body muscles.

P : Pseudocoel.

T : Tegument.

U : Uterus.

UB : Uterine bell.

UBP : Uterine bell pore.

V : Vagina.

VLS : Ventral ligament sac.

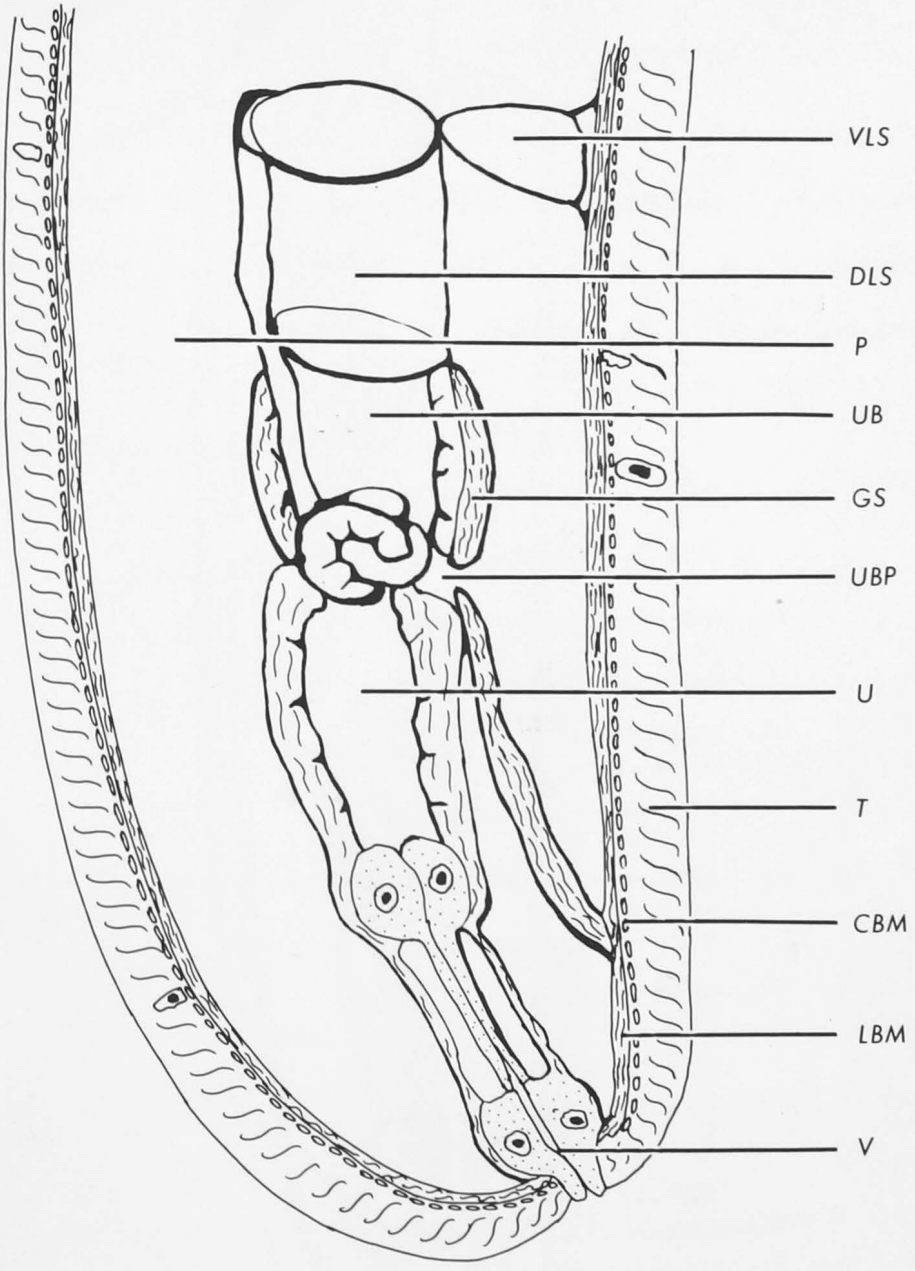

VLS
DLS
P
UB
GS
UBP
U
T
CBM
LBM
V

FIGURE 1.4.

Diagrammatic representation of the reproductive system of male M. dubius. (Modified after King (1965), and reproduced with the permission of the author).

B : Bladder.

BM : Bursa musculature.

CBM : Circular body muscles.

CD : Cement duct.

CG : Cement gland.

DLS : Dorsal ligament sac.

GS : Genital sheath.

LBM : Longitudinal body muscles.

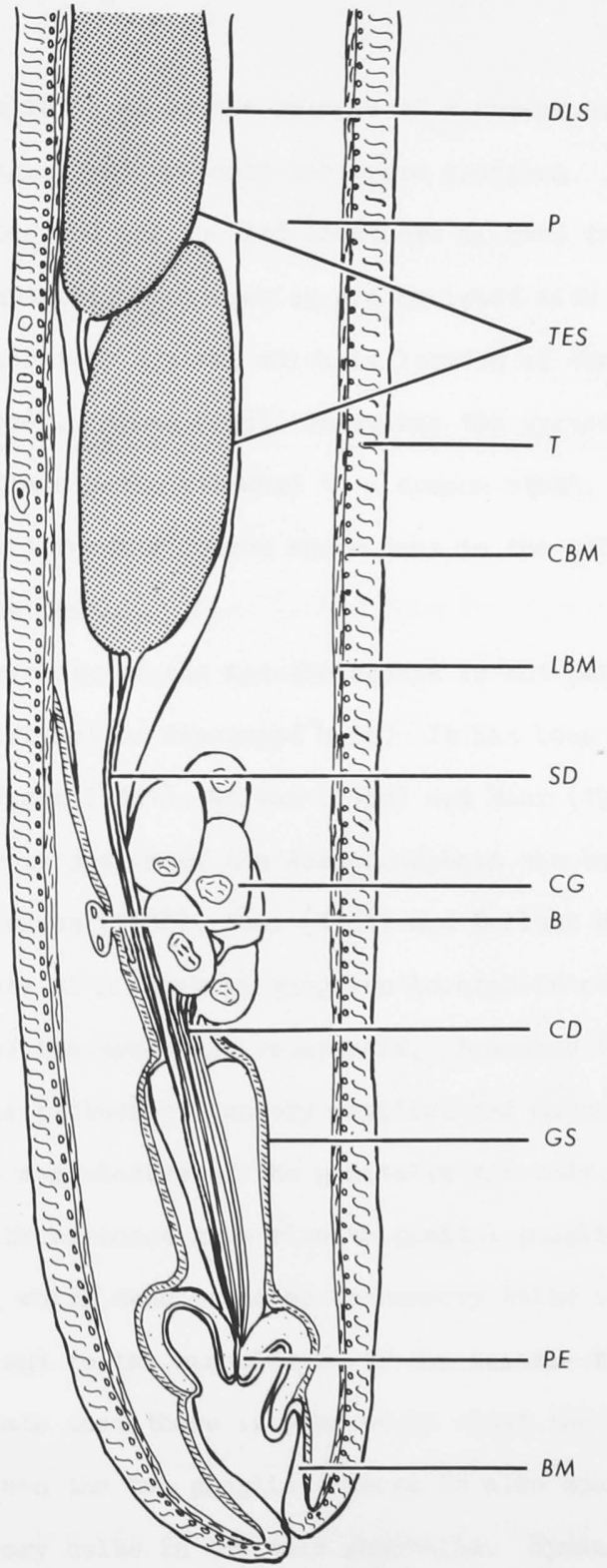
P : Pseudocoel.

PE : Penis.

SD : Sperm duct.

T : Tegument.

TES : Testes.


DLS
P
TES
T
CBM
LBM
SD
CG
B
CD
GS
PE
BM

released. This is followed by the release of a cement secretion which plugs the vagina and prevents the sperm escaping. The plug is subsequently shed and the shelled acanthors allowed to escape.

Some acanthocephalan species are equipped with a protonephridial excretory system, which is located at the posterior end of the pseudocoel. Hyman (1951) describes the system as "... a branched mass of flame cells attached to a common stem". The stem or collecting duct joins the common sperm duct in the male, or the uterine tube in the female.

The embryology of the Acanthocephala is not pertinent to this thesis and will not be discussed here. It has been reviewed by Meyer (1932), Hyman (1951), Golvan (1958) and Baer (1961).

The nervous system of the Acanthocephala has been reviewed by Hyman (1951), Golvan (1958), Baer (1961) and Bullock and Horridge (1965). It consists of a cerebral ganglion located in contact with the ventral wall of the proboscis receptacle. Branches from this ganglion run to the proboscis, sensory papillae and musculature, and posteriorly to the musculature of the genitalia and body wall. In males, the posterior branches give rise to genital ganglia at the base of the penis, which send branches to sensory bulbs in the male genital apparatus and to the musculature of the bursa. Bullock and Horridge (1965) state that there is some doubt about the existence of the nerves between the two ganglia. There is also doubt about the nature of the sensory bulbs in the male genitalia. Hyman (1951) states that they are usually considered to be tactile. Bullock and

Horridge suggest that they may be chemoreceptors.

More detailed descriptions of the morphology of the parasite used for these studies, Moniliformis dubius, will be given in the introduction to the relevant chapter.

1.4. The History and Maintenance of the Experimental Infection.

1.4.1. The History of the Infection.

Burlinghame and Chandler (1941) were the first to establish a laboratory colony of Moniliformis dubius. The worms used by the author were maintained in white rats, and the American cockroach, (Periplaneta americana) by the same methods.

The Moniliformis dubius strain used throughout this work was isolated in 1958 by Dr. S.J. Edmonds of the Zoology Department of the University of Adelaide, from wild rats caught in Brisbane. The culture was established at the Zoology Department of The Australian National University in February, 1962 by Dr. W.L. Nicholas from infected cockroaches received from Dr. Edmonds. Since its establishment the culture has been maintained in albino laboratory rats and a laboratory culture of the cockroach, Periplaneta americana.

1.4.2. The Maintenance of the Infection.

The infection has been artificially maintained by feeding cockroaches and rats on concentrated infective materials. Until

1965 cockroaches were infected by placing pseudocoel fluids, extracted by chopping fresh worms in Ringers solution, in bottles containing cockroaches which had been starved for 24 hours. This method of infecting cockroaches produced extremely variable but consistently poor infections. King (1965) described a method of infecting individual cockroaches with a Pasteur pipette, which was adopted and proved far more successful.

Rats were starved for at least twelve hours before they were infected with ten cystacanths on a piece of bread. The cystacanths were extracted by cutting up infected cockroaches in Ringers solution and collecting the freed cystacanths with a Pasteur pipette. The infections which resulted were very variable. King (1965) used a stomach tube to infect rats, but found that the infections which resulted were just as variable as those obtained by an infective feed. Burlinghame and Chandler (1941) showed that M. dubius would only become established in the rat gut if they attached to the gut wall between 10 and 60 cms. down from the pyloric sphincter. They also showed hereditary differences in susceptibility between rats of individual litters. These two findings could explain the variable nature of the infections.

Burlinghame and Chandler (1941) and Holmes (1961) investigated the effects of overcrowding of worms in the intestine and competition between worms for nutrients, and determined that both these factors reduced the number and size of worms in the gut. The author tested the harvest of worms obtained from several age groups

of rats, and found that the larger the rat the larger the number of worms that became established. This is consistent with the findings of both Holmes, and Burlinghame and Chandler. The latter also showed that M. dubius produces no demonstrable immune reaction in the rat. Thus, older, and presumably immunologically more competent rats, would not show any increased resistance to the establishment of M. dubius infections. For these reasons the oldest rats available were always used for the maintenance of worm stocks.

Rats were housed in groups of five in 17" x 13" x 5" deep steel tins containing wood shavings and covered by wire clip-on tops equipped with water and food containers. Food and water ad libitum were provided for all stock rats. Tins of infected rats were labelled with the sex of the rat and the date of infection.

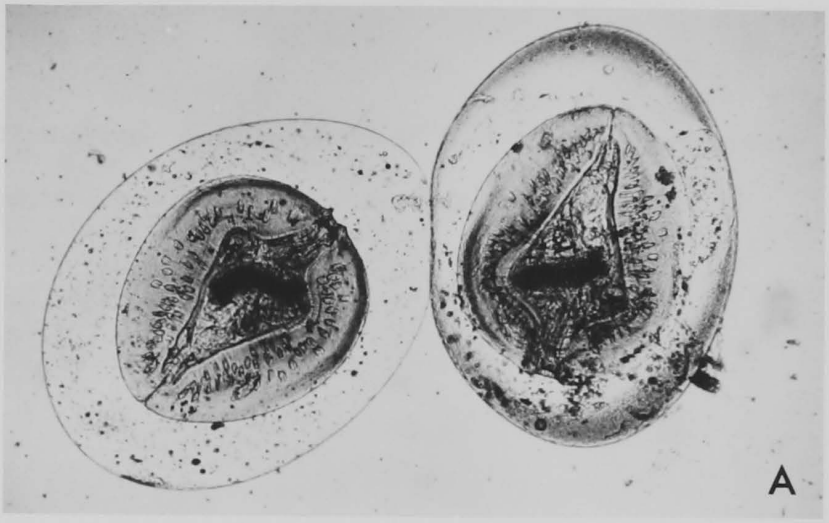
Infected cockroaches were maintained in 2 lb. preserving jars equipped with gauze lined ventilated tops containing unlimited food (commercial rat cake) and water. The cockroaches were maintained at a temperature of 25-27^{o}C in a thermostatically controlled room.

No difficulty was experienced in maintaining stocks of M. dubius in rats or cockroaches until June, 1966 when the cockroaches began to produce "activated" cystacanths of the type shown in plate 1.1. These "activated" cystacanths have everted probosces and have assumed a moniliform shape. They are not infective to rats. It was found that the temperature control unit had failed, allowing the

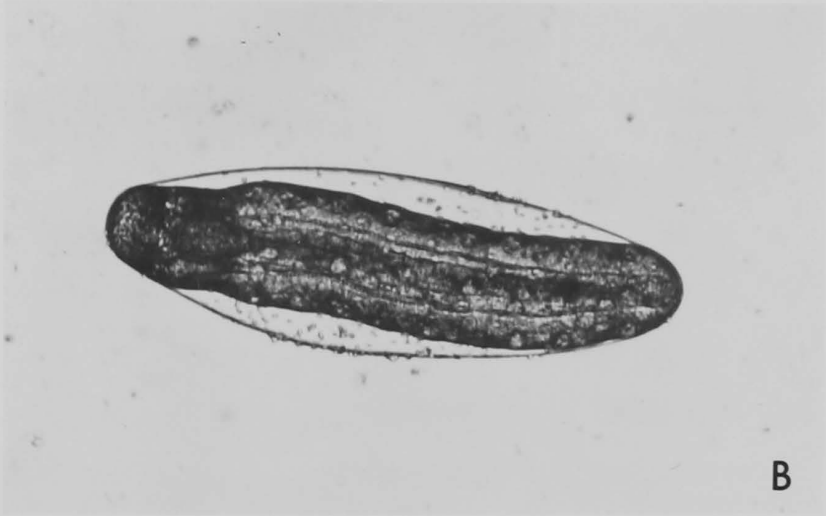
PLATE 1.1

A. Normal infective cystacanth from the haemocoel of the cockroach Periplaneta americana.

B and C. Non-infective "activated" cystacanths from the haemocoel of the cockroach Periplaneta americana. The specimen shown in C is a later stage of that shown in B.



A



B

C

temperature of the room to rise to 37-38°C at night. Once proper temperature control was achieved no further difficulty was experienced.

CHAPTER 2

THE OSMOTIC RESPONSES OF MONILIFORMIS DUBIUS (ACANTHOCEPHALA)

2.1. Introduction.

The fine structure of the body wall of M. dubius has been described from electron micrographs by Nicholas and Mercer (1965). It consists of an outer, non-living, fibrous mucopolysaccharide layer, the epicuticle, which is about 1μ thick. This overlays a typical double plasma membrane which in turn covers a sub-plasma membrane similar to the first. The striped layer consists of canals, 3.3μ deep penetrating the plasma membrane and giving rise to membrane bound vesicles which ramify throughout the body wall. The two membranes and the pores of the canals can be seen in plate 2.1. The body wall is a cytoplasmic syncytium of fibres interspersed with mitochondria, Golgi complex, endoplasmic reticulum and many lipid inclusions.

The lacunar canal system of the Acanthocephala has only been described in Neoechinorhynchus rutili, Meyer (1931). It is a ramifying system of canals, the larger of which have walls composed of the radial fibres of the tegument. The smaller canals are not lined in such a characteristic manner, and the fibres of the body wall themselves represent the finest canals of the lacunar system.

The inner boundary of the body wall is a double-layered basement membrane. The cytoplasm of the body wall in the vicinity

PLATE 2.1

Electronmicrograph of the surface of M. dubius

(x 73,200)

P : Pore.

PM : Plasma membrane.

SPM : Sub-plasma membrane.


P
PM
SPM

of the basement membrane is interspersed with frequent vesicles, some of which were seen coalescing with, or detaching from, the basement membrane. The circular and longitudinal muscles of the body wall are attached below the basement membrane.

The body wall of an acanthocephalan parasitic in the duck, Polymorphus minutus, has been described by Crompton and Lee (1965). It possess structures and organelles similar to those of M. dubius but these authors suggest that the canals of the striped layer may communicate with the lacunar canals found deeper in the body wall. However, Stranack, Woodhouse and Griffin (1966) working with Pomphorhynchus laevis, an acanthocephalan parasite of fresh water fish, found canals at the level of the basement membrane, and suggest that the canals of the striped layer may ramify through the body wall and communicate with them.

Thus if the various species may be directly compared there is some doubt about the extent and continuity of the canal systems of the Acanthocephala. All the species described possess a plasma membrane as a living bounding layer. Some of the acanthocephalan species described also possess a mucopolysaccharide epicuticle which is the bounding layer of the tegument. This structure is fibrous and does not have the supporting and protective characteristics of the nematode cuticle. A convoluted basement membrane has been described for the three species, and has been compared to the ion exchange surfaces of the proximal convoluted tubule of the mammalian kidney by Stranack, et al. A greater understanding of the absorptive

processes of the parasite may be gained from a study of the permeability properties of the bounding membranes.

Data will be presented in chapter 3 to show that M. dubius maintains a high concentration of K, and a low concentration of Na, in the body wall. This distribution of cations is similar to that observed in mammalian tissues. The reason why many cells accumulate higher concentrations of K than Na is not understood. Glynn (1959b) advances three possible reasons for the evolution of this cation distribution. The first is that some enzymes may require high concentrations of K and low concentrations of Na for optimal activity. Secondly, any cell or tissue which retains proteins and other large molecules, but is permeable to salts and water, will face the problem of water inflow, as the permeable salts redistribute themselves in a Gibbs-Donnan equilibrium. This effect may be overcome by actively pumping out Na ions, in effect making the cell impermeable to them, so that a double Donnan equilibrium is achieved. K, the major cation is retained within the cell, and the diffusible anions, primarily Cl, are passively distributed to maintain electrical neutrality. Bayliss (1960) gives the permeability to Na of nerve and muscle membranes of mammals as 0.1 to 0.05 of the permeability of K ions, as determined by radioactive tracers. The low concentration of intracellular Na is attributed to the active extrusion of the Na ion by the cell membranes. The third reason advanced concerns cell excitability, which depends on an unequal distribution of Na and K ions. These three reasons may, however, be either causes or effects

of cation balances, which are maintained between cells and tissue fluids.

Evidence will be presented later suggesting that active accumulation of cations takes place in M. dubius, so a brief review of the characteristics of membrane-sited cation pumps in other tissues is warranted.

It was postulated by Bernstein (1902) (quoted by Katz (1966)) that the cell membrane is permeable only to K ion and completely impermeable to Na ion and anions. This concept explained the imbalance of cations and the electrical inequality between the cell and its environment in most animals.

Ussing (1960) has demonstrated permeability differences between the outer and inner surfaces of the frog skin and the existence of a Na : K exchange pump, which requires the presence of oxygen for its operation. Conway (1960) reviewed the literature on cation pumps and their significance in muscular tissues, and Eccles (1957, 1964) published two volumes on the physiology of nerve fibres and synaptic transmission. A vast literature on biological transport has developed postulating the existence of energy dependent cation pumps, which may or may not be Na : K exchange pumps. The work of Cross, Keynes and Rybova (1965) and a review by Whittam (1963) dealt with the energetics of these processes. Ussing (1957, 1965), Conway (1960), Glynn (1959a, 1959b) and Hokin and Hokin (1963) have all reviewed the evidence for the existence of, and possible mechanisms of, cation pumps.

Garrahan and Glynn (1967a) showed that the ouabain-sensitive Na : K exchange pump in red blood cells became an ouabain-sensitive Na : Na exchange pump in the absence of external K. Thus, the exchange pump is relatively non-specific, at least with respect to inward transport of Na and K. They also showed that there is a progressive decrease in Na : Na exchange and increase in Na : K exchange as the external K concentration increased. External K at physiological concentrations produces no Na : Na exchange. Thus the exchange pump preferentially transports K inwards but is capable of exchanging Na.

Ion binding could explain the retention of high concentrations of K within cells. Glynn (1959a) examined this possibility and concluded that it could not account for the observations of various workers on cation fluxes and high anion concentrations within the cell. He gives a maximum figure of 16% for bound cell water and points out that if the intracellular K is bound there would need to be a very much higher proportion of the cell water bound to maintain osmotic equilibrium.

Other types of ion transport systems have been located. Curran (1960) presented evidence for a Cl transport system in the rat ileum. This system is dependent on a source of carbohydrate energy and is inhibited by 2, 4 - dinitrophenol. Evidence for the existence of other types of anion transport systems is furnished by Sutcliffe and Hurd (1959) who investigated active accumulation of HCO_3 ion by vacuolated plant cells, and Wolff and Maurey (1961) who

demonstrated the accumulation of I by thyroid tissues. Transport of I is dependent on the presence of K ion, and the inhibition of I transport by cardiac glycosides is attributed to the inhibition of the K transport system. This evidence suggests that coupled anion-cation transport mechanisms may exist. A Cl ion transport system was reported by Bielawski (1964) who reported its presence in crayfish gills, which transported Cl ion into the tissues against a concentration gradient. This system was inhibited by high internal concentrations of Cl ion, indicating that feed-back control mechanisms may exist to moderate its activity. Rehm (1950) reviewed the literature on the formation of HCl by the stomach and proposed a complex mechanism whereby established electromotive forces produce H ion with the secretion of Cl ion by current counterflow.

Other types of biological transport systems have been suggested which transport amino acids and sugars in the intestine of some animals and parasites.

Transport of a substance is defined by Csáky (1965) to be active if it takes place against an electrochemical concentration gradient, in the case of electrically charged molecules, or a chemical concentration gradient, in the case of electrically neutral molecules. Thus the demonstration of an active transport process requires both knowledge of the chemical concentrations of the solute under consideration on both sides of the membrane through which active transport is to be demonstrated, and the magnitude and polarity of any electrical potential exerted by the distribution of

all the solutes.

Tosteson (1963) points out that most mammalian cells have an internal hydrostatic pressure which is equal to the pressure of the extracellular fluids. Thus the activity of water in both phases is the same. This result is achieved by the actively maintained differences in concentrations of Na and K, and the passively maintained distribution of Cl ion across the membrane, to distribute the diffusible and non-diffusible cell contents in a double-Donnan equilibrium. The cell volume is then controlled by the activity of the cation transport mechanisms (Potts and Parry (1964)). This thesis is not concerned with the precise mechanisms of these pumps or their kinetics. It seeks merely to establish whether or not cation and amino acid active transport systems exist in the acanthocephalan Moniliformis dubius.

The structure of the cell membrane, as postulated by Davson and Danielli (1952) consists of a double layer with a core of lipoid material oriented with its polar ends facing outwards and its nonpolar ends facing towards one another. The polar groups are associated with a layer of protein. The thickness of this arrangement was estimated to be 75 $\mathring{A}$. Willmer (1961) reviewed the postulated compositions and structures of membranes. They are primarily composed of closely packed C_{16} - C_{22} hydrocarbons, which may be present as neutral fats, fatty acids and their esters, steroids and their esters, but mainly as phospholipins, in one or more monomolecular layers. The long aliphatic chains of the

phospholipins are in close parallel array, with the long hydropobic hydrocarbon chains packed together, and the hydrophilic end of the molecules facing outward. This type of arrangement had been suggested by Davson and Danielli (1943), Frey-Wyssling (1953) and Finean (1953). The arrangement of the phospholipins as single, double or multiple layers, and whether or not they have protein sheets attached to them, and whether or not the fatty layers are separated by aqueons layers may vary from membrane to membrane and may vary within the same membrane at different times. Winkler and de Jong (1941) consider the surface membrane of the erythrocyte to be basically a lipoid layer attached to the oriented proteins of the red cell stroma. Davson and Danielli (1943) discuss two protein layers surrounding a fatty core while Finean and Robertson (1958) elaborate this scheme and suggest a similar structure for the repetitive layers of myelin. Many of the observations of membranes come from electron microscopy although this data is obtained from an artifact of osmium fixation, the results are consistent with the structure of a lipo-protein layer. The interpretation must remain, however, uncertain.

Willmer (1961) discussed the results obtained by forming artificial lecithin membranes on water. If cholesterol molecules are interspersed with a lecithin monolayer the structure is stabilised. He suggested on the basis of this type of experiment that membranes probably consist of lecithin and cholesterol. Parport and Ballentine (1952) who analysed the red cell ghost

suggested a lipid to protein ratio of 1.7 to 1.0. The protein was fibrous in nature. The lipids were 15 - 32% cholesterol and 55 - 75% phospholipids, mainly lecithin and cephalin. Davson and Danielli (1952) suggested that membranes are covered by a protein layer because the surface tension of some egg cell membranes is too low for a lipid surface. Giese (1960) presents more direct evidence for the postulated arrangement of lipoid and protein from X-ray diffraction and polarised light studies. He cites Waugh and Schmitt (1940), who suggested from studies with polarised light that the lipids of the plasma membrane are radially arranged and the proteins parallel to the cell surface. The authors state however that other interpretations of the data are possible. Schmitt et al (1940) determined a similar structure using X-ray diffraction. Hokin and Hokin (1965) suggest that the phospholipid of cell membranes is a fatty acid with hydrophilic glycorol phosphate attached to one end.

Lucy (1964) postulated that the membranes may be composed of globular lipid micelles, 40 - 50 Å in diameter, and overlaid by protein or mucopolysaccharide layers. Lucy contends that differentiation of micellar membranes from leaflet membranes would be impossible with the electron microscope. He discusses the insertion of proteins, enzymes etc. into the structure and the packing of the micelles to produce hydrated pore canals. Leaflet-micellar transitions are also postulated. However, the most widely accepted view of membrane structure is the lipoid protein leaflet of the type originally postulated by Davson and Danielli (1952).

Davson and Danielli refer to the capacity of many membranes to act as molecular sieves. Collander (1937) proposed the lipoid-pore membrane, and suggested that lipid-soluble compounds would penetrate the membrane by dissolving in the membrane phase, while lipid-insoluble compounds, particularly polar compounds, penetrate the hydrated canals or pores in the structure. Danielli (1954a) suggested that protein lined pores may penetrate the lipoid layers to produce hydrated canals. Hillier and Hoffman (1953) suggested that the plasma membrane of the erythrocyte may be composed of a protein mesh which contains the lipid. This structure also provides regions free of lipid for the formation of a continuous water phase.

Panagelli and Solomon (1957) have estimated the pore radius of the human red blood cell at 3.5 $\mathring{A}$ and Goldstein and Solomon (1960) estimated 4.2 $\mathring{A}$. Conway (1954) gives the average diameter of membrane pores in soft body tissues as approximately 8 $\mathring{A}$, while Solomon (1960) gives a representative figure of 7 $\mathring{A}$ for membrane pores. The bi-layer structure and pores, give membranes their properties of semipermeability.

Evidence for the existence of cation pumps in M. dubius will be presented in section 2.3. This evidence is based on the response of M. dubius to various incubation media. For comparative purposes a review of similar work with other animals is included here.

Schopfer (1924, 1925) investigated the response of Ascaris

sp. to hypotonic and hypertonic solutions. He states that the response of the parasite indicates that the cuticle shows properties of semipermeability, but adds that this remains to be proved. Wardle (1937) investigated the response of Moniezia expansa to various saline solutions and found that saline solutions which were isotonic with mammalian tissues were also isotonic for this parasite. Wardle and Schopfer obtained a similar response when Moniezia was placed in hypertonic and hypotonic solutions, as both parasites lost weight in hypertonic solutions and gained weight in hypotonic solutions. Both authors interpret these results in terms of gain or loss of water, and regard it as a logical consequence of the osmotic gradient established by the bathing solution. Wardle conducted his experiments in a variety of saline solutions over 6 hours. His published results show that the worms had remained at a higher or lower weight over this period, indicating that the bounding membrane had successfully resisted the massive movement of solutes down the concentration gradients established by the bathing medium. Schopfer (1925) obtained a similar result for Ascaris over 18 hours in distilled water and 10 hours in 16% NaCl.

Schopfer (1924, 1925) considers that the water exchange he observed in ligatured Ascaris took place through the cuticle and not through the uterus. Nor was the increased weight of water in the gut of the worm responsible for the total weight increase. Hobson, Stephenson and Beadle (1952) performed the same experiment with Ascaris lumbricoides ligatured at the anterior end. They conclude

that the gut is an important water exchange surface. Schopfer (1926) tested the response of Ascaris sp. to dilutions of pig intestinal fluid. The results he obtained are the same as those from NaCl solutions. Schopfer comments on the asymptotic nature of the curve obtained when Ascaris is swollen in hypotonic media. He considers that the asymptote is reached when the hydrostatic pressure exerted by the stretched body wall is equal to the osmotic pressure difference between the worm and the solution.

Fairbairn (1957) reported that Ascaris and Parascaris sp. do not reverse their tendency to swell or shrink if they are left for several days in hypotonic or hypertonic solutions.

These results indicate that the body wall of Moniezia, Ascaris and Parascaris spp. possess semipermeable capabilities. Hobson, Stephenson and Beadle concluded that the cuticle of A. lumbricoides is readily permeable to Cl ion but that the body wall is capable of active outward transport of Cl ion. Harris and Crofton (1957) determined that the internal turgor pressure of Ascaris fluctuated between 16 and 125 mm Hg, with a mean value of 70 mm Hg. These workers described a fibrous lattice structure in the body wall of Ascaris which maintained the internal turgor pressure and a constant tension on the longitudinal body muscles. They consider that the internal turgor pressure was the skeleton around which the muscles of Ascaris operate.

Hammond (1966) described the muscular activity of Acanthocephalus ranae as unitary because the longitudinal and

circular body muscles can only operate antagonistically. For this reason the body may be short and of large diameter or long and narrow. M. dubius freshly removed from the rat gut is flaccid and shows no evidence of a rigid hydrostatic skeleton.

Siddiqi and Lutz (1966) investigated the osmotic responses of Fasciola gigantica. They determined that this fluke is an imperfect osmometer, as it loses weight in hypertonic solutions and gains weight in hypotonic solutions. The fluke will regain its initial weight if transferred from a hypo- or hypertonic solution to Tyrodes balanced salt solution, showing that F. gigantica is isotonic with Tyrodes solution. These authors conclude that water enters the body more readily than it leaves as the increased weight gain with increasing hypotonicity is greater than the increased weight loss as hypertonicity increases. Knox and Pantelouris (1966) found a similar differential rate of inward and outward water movement in Fasciola hepatica. All these authors conclude that the flukes act as simple but imperfect osmometers, adjusting to changes in the tonicity and molarity of their environment by passive leakage of salts and water in both directions.

Nicholas and Grigg (1965) investigated the conditions under which M. dubius would survive in vitro. They used Hanks balanced saline and Eagles medium plus serum. Eagles medium plus serum, which is isotonic with mammalian tissues, enabled the worms to maintain osmotic control for several hours after which they swelled irreversibly.

Gettier (1942) investigated the saline requirements of Neoechinorhynchus emydis. He determined that solutions isotonic with 0.5% (= 85.4 mM) NaCl were the most favourable. He tested survival times in various single inorganic chloride solutions and ranked them as follows: NaCl : $CaCl_2$: $MgCl_2$: KCl. The best survival was obtained in a modified Ringers solution isotonic with 0.5% NaCl. Thus a solution isotonic with 85 mM NaCl would appear to be the most osomotically favourable medium for this acanthocephalan parasite from the turtle.

Van Cleave and Ross (1944) studied the response of Neoechinorhynchus emydis to hypotonic solutions. In water N. emydis rapidly swelled and died. They determined that 0.8 - 0.85% NaCl (137 - 145 mM NaCl) was required for these worms to retain their normally flattened shape. If the worms were first swollen in tap water and then transferred to 0.85% NaCl they recovered their flattened shape but eventually swelled again. The same result was obtained if the worms were transferred to a hypertonic medium from tap water. Van Cleave and Ross examined the response of the worms in situ and found that swelling occurs if the intestine is flooded with water.

These results indicate that M. dubius, a parasite of mammals and N. emydis, a parasite of a non-mammal, are isotonic with solutions isotonic with mammalian tissues, i.e. 145 mM NaCl. Gettier's result appears very low and cannot be reconciled with that of Van Cleave and Ross (1944).

Harms (1965) attempted the *in vitro* cultivation of the acanthocephalan, *Octospinifer macilentis*, a parasite of the common sucker, *Catostomus commersoni*. He determined that 0.85% NaCl (145 mM NaCl) was isotonic for this species from a non-mammal host.

The concept of activation energy is used in section 2.3.3. Activation energy is the energy, usually kinetic, which a molecule or molecules must possess to initiate a reaction or event. (Bayliss (1959)). Activation energy is usually derived from the kinetic energy of the molecules involved in the reaction, and as kinetic energy is directly related to temperature, reducing the temperature may reduce the available energy of the reactants to such an extent that the reaction or event does not occur.

The series of experiments reported below seek to establish the semipermeable nature of the tegument of *M. dubius*.

2.2. Materials and Methods.

The worms used in the experiments reported in this thesis were collected from rats which were killed by a blow on the head. They were used immediately, except where an equilibration period was allowed. The nature and duration of the equilibration period is reported with the relevant experiment.

The response of *M. dubius* to the test solutions was determined by weighing the worms at various time intervals to ascertain whether or not any weight change had occurred. Any change

in weight was considered to represent a gain or loss of water. Worms were harvested from freshly killed rats and wiped between the fingers to remove any ingesta which was attached. The worms used were sexually mature males and females 6 - 8 weeks of age, which were obtained from mixed sex infections in male and female rats fed on a commercial rat cake. They were weighed on a Mettler automatic balance, the reading being taken from the balance immediately the scale reading had stabilised. If the worms were left on the balance pan a progressive weight loss occurred as the surface dried. The worms were transferred, on a wire hook, to 30 ml of the test solution immediately after weighing. Forceps and other handling devices which apply pressure to the worm were avoided at all times. If it was necessary that the worms be manipulated, it was done, whenever possible, with the fingers. After the desired time interval the worm was removed from the bathing solution with the wire hook, blotted on an absorbent tissue and weighed. The solutions and conditions used are given with the results in section 2.3.

All the solutions used in the experiments were prepared in glass using water obtained from a metal still, except those in chapter 3 where additional purification was required.

Throughout the thesis repeated reference is made to Hanks balanced salt solution. Except where otherwise stated the Hanks used was prepared according to the method of Penso and Balducci (1963). It is an aqueous medium containing 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4.7\ H_2O$, 1 mM Na_2HPO_4, 1 mM KH_2PO_4, 4 mM $NaHCO_3$ and 11 mM

glucose.

Osmotic pressures of all solutions were estimated from the following formula given by Diem (1962):

Osmotic Pressure (ideal) = 0.08205 x T x v x M x 760 mm Hg.

where 0.08205 is the gas constant in litre atmospheres, T is the absolute temperature in ^{o}K (= ^{o}C + 273), v is the number of particles formed by complete dissociation of the solute and M is the moles of undissociated solute per 1000 g of water i.e. the molality. The real osmotic pressure is given by:

Real osmotic pressure = Ideal osmotic pressure x the osmotic coefficient, g.

g is taken from the tables given by Diem (1962) and varies inversely with solute concentration.

2.3. Results.

2.3.1. A series of experiments was undertaken to determine the response of M. dubius to hyperosmotic and hypo-osmotic NaCl and KCl solutions. The results of incubation of male and female worms in 30 mls of (1) 342 mM NaCl, (2) 342 mM KCl and (3) distilled water at 37^{o}C are shown graphically in figure 2.1. The per cent change in wet weight of the worms with time was calculated from:-

$$\frac{\text{Weight at time } t_1 \text{ - original weight}}{\text{Original weight}} \times \frac{100}{1}$$

FIGURE 2.1.

The average per cent wet weight change of male and female M. dubius incubated in 342 mM NaCl, KCl and distilled water at 37°C.

(All lines drawn by inspection).

The symbols represent:

Female - distilled water

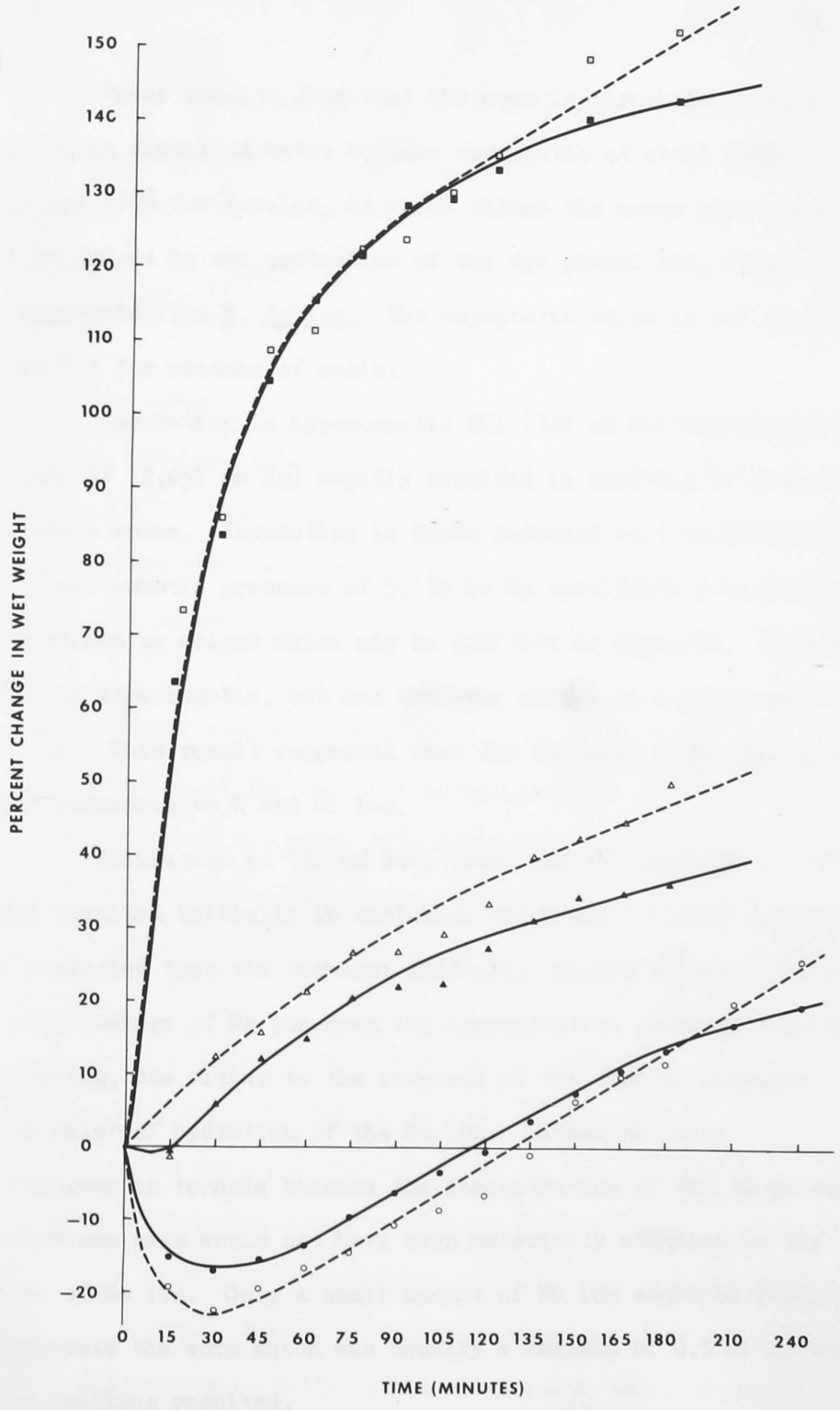
Male - distilled water

Female - 342 mM KCl

Male - 342 mM KCl

Female - 342 mM NaCl

Male - 342 mM NaCl


PERCENT CHANGE IN WET WEIGHT
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
−10
−20
0
15
30
45
60
75
90
105
120
135
150
165
180
210
240
TIME (MINUTES)

These results show that the worm is permeable to water. Swelling in distilled water becomes asymptotic at about 180% for males and 160% for females, at which values the worms were usually dead as judged by the pentration of the dye phenol red, which does not penetrate live M. dubius. The asymptotic value is not shown in figure 2.1 for reasons of scale.

Incubation in hyperosmotic KCl (342 mM KCl has an osmotic pressure of 12,057 mm Hg) rapidly resulted in swelling of both male and female worms. Incubation in Hanks balanced salt solution, which has a real osmotic pressure of 5,739 mm Hg resultedin a very small fluctuations in weight which may be positive or negative. Thus 342 mM KCl is hyperosmotic, but has the same effect as a hypo-osmotic solution. This result suggested that the tegument of M. dubius was freely permeable to K and Cl ion.

Incubation in 342 mM NaCl (real osmotic pressure 12,057 mm Hg) resulted initially in shrinkage which was followed by swelling. This suggested that the tegument initially imposed an osmotic barrier and then leakage of Na ion down its concentration gradient resulted in swelling, due either to the reversal of the osmotic gradient, or to the water of hydration of the Na ion. Either of these alternatives is tenable because the concentration of the 30 ml volume of solutions used would not have been materially affected by the removal of Na ion. Only a small amount of Na ion would be required to penetrate the worm which was usually a maximum of 0.3 ml in volume, before swelling resulted.

Thus the worm appeared to be an imperfect osmometer. If the semipermeable barrier of M. dubius were equipped with pores through which the hydrated ions could penetrate they would appear to be about 8 Å, the same diameter as those found in muscle membranes, because of the more rapid penetration of K ion relative to Na ion.

2.3.2. In order to determine whether or not any metabolic control was exerted over the penetration of Na and K ions the experiment was repeated at 4°C. The concentrations of the salt solutions were calculated from the ideal osmotic pressures of 342 mM NaCl and KCl at 37°C. The relationship between osmotic pressure and temperature is:-

real osmotic pressure = 0.08205 x T x v x m x 760 x g mM Hg.

The concentration of the osmotically equivalent solutions was determined by substitution in this equation. The resulting 386 mM solutions then have the same ideal osmotic pressure at 4°C as the 342 mM solutions at 37°C. The results of this experiment are shown in figure 2.2.

These results clearly show that a reduction in incubation temperature to 4°C has largely prevented the penetration of Na and K ions. The worms were all gaining weight in the hyperosmotic solutions after 3 hours having previously lost weight. This may be attributed to passive penetration of these ions and the resulting increase in internal osmotic pressure causing an inflow of water. The rate of swelling in distilled water has also been reduced. This

FIGURE 2.2.

The average per cent wet weight change of male and female M. dubius incubated in 386 mM NaCl, KCl and distilled water at 4°C.

(All lines drawn by inspection).

The symbols represent:

Female - distilled water

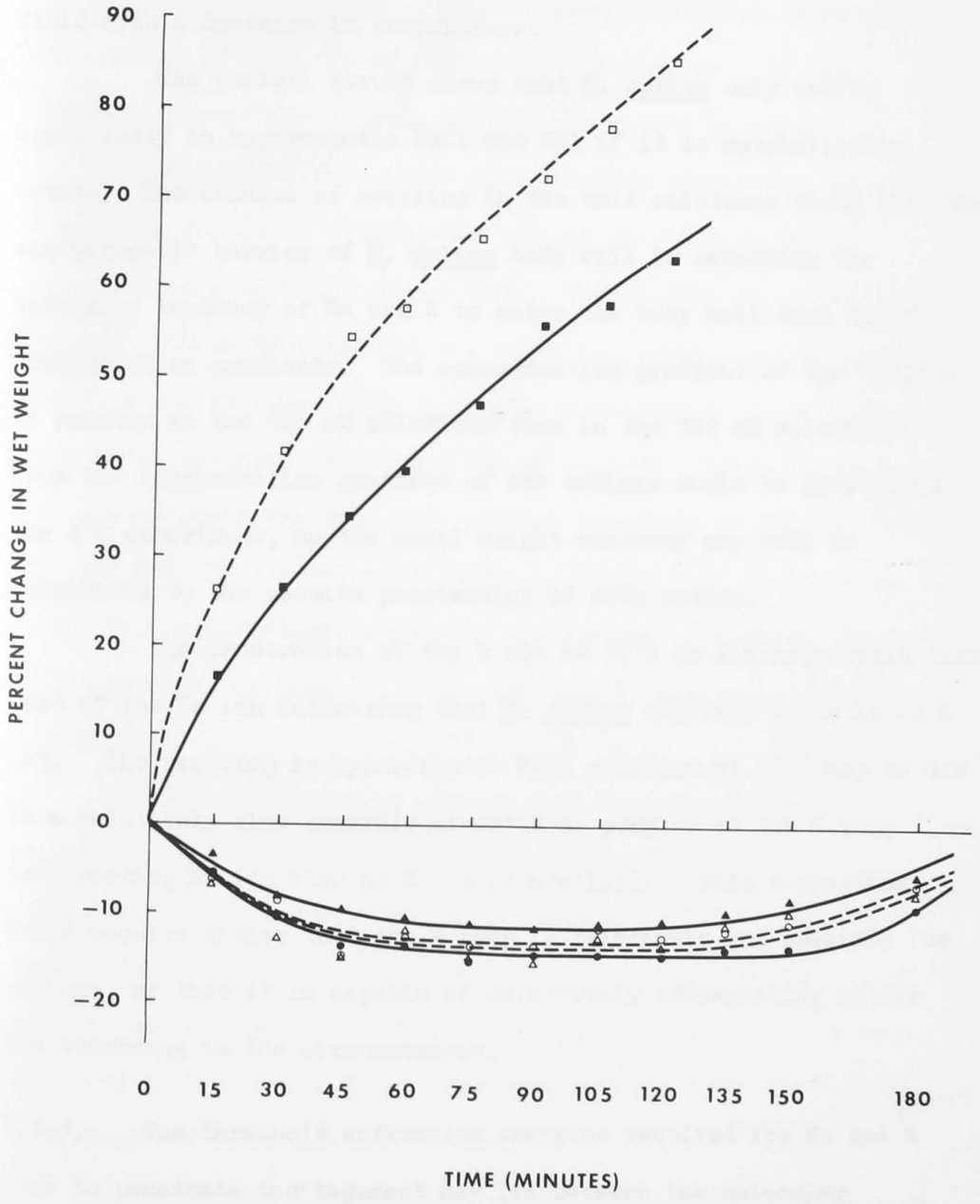
Male - distilled water

Female - 386 mM KCl

Male - 386 mM KCl

Female - 386 mM NaCl

Male - 386 mM NaCl


90
80
70
60
50
40
30
20
10
0
−10
−20
PERCENT CHANGE IN WET WEIGHT
0
15
30
45
60
75
90
105
120
135
150
180
TIME (MINUTES)

may be attributable to the decrease in osmotic pressure of the worm fluid with a decrease in temperature.

The overall result shows that M. dubius only swells appreciably in hyperosmotic NaCl and KCl if it is metabolically active. The absence of swelling in the salt solutions shows that the semipermeable barrier of M. dubius body wall is resisting the diffusive tendency of Na and K to enter the body wall down their concentration gradients. The concentration gradient of the cations is greater in the 386 mM solutions than in the 342 mM solutions. Thus the concentration gradient of the cations would be greater in the 4°C experiment, so the small weight recovery may well be attributed to the passive penetration of some cation.

The penetration of the K ion at 37°C is far more rapid than that of the Na ion indicating that M. dubius actively accumulates K ion. The swelling in hyperosmotic NaCl solution at 37°C may be due to a relatively slow inwardly directed Na pump or to the K pump transporting Na ion when no K ion is available. This suggestion would require either that the K pump is relatively non-specific for cations, or that it is capable of selectively transporting either ion according to the circumstances.

2.3.3. The threshold activation energies required for Na and K ions to penetrate the tegument may lie between the molecular energies available at 37°C and 4°C. If this were so no penetration of the Na and K ions would be expected at 4°C regardless of the

physiological state of the worm. To check this point worms were incubated at 37°C in 190 mM NaCl and KCl solutions. If the swelling in hyperosmotic NaCl and KCl was caused by passive diffusion of cations down their concentration gradients, the degree of swelling should be considerably reduced by reducing the concentration gradient into the worm. The results of incubating M. dubius in 190 mM NaCl and KCl are shown in figure 2.3. In order to determine the response of M. dubius to solutions of large molecules, worms were incubated in 684 and 380 mM sucrose. These solutions have respectively the same real osmotic pressures as 386 and 190 mM NaCl and KCl. The results are shown in figure 2.3. Sucrose was chosen as a non-charged relatively impermeable substance. Data will be presented in section 6.3.1 to show that M. dubius is not permeable to sucrose.

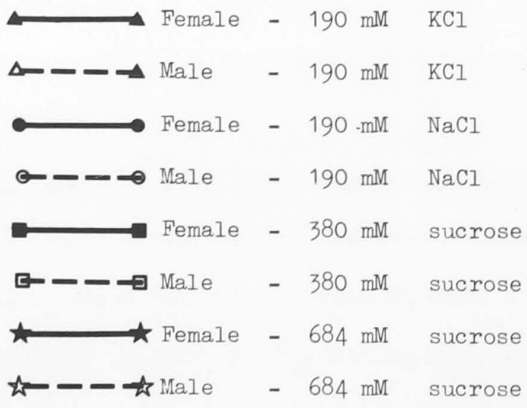
The experiments in sucrose show that the body wall of M. dubius is reasonably semi-permeable, as the worms have not continually lost weight through loss of cations down their own concentration gradients, nor gained weight through penetration of sucrose, unless exactly the same osmotic equivalent of salts was exchanged for sucrose. This result suggests that the membrane allows the passage of water but not solutes.

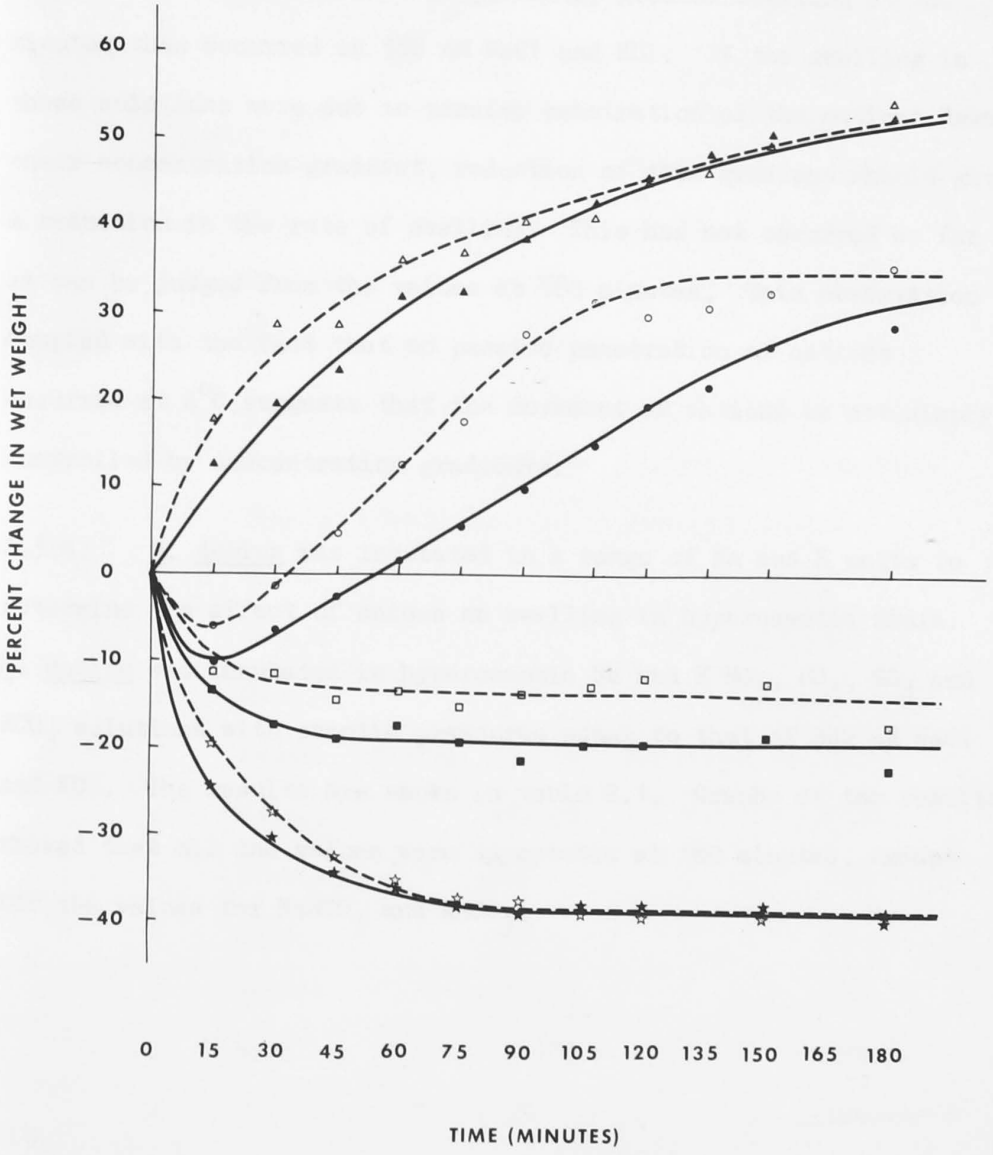
Figure 2.3 shows weight change curves for 380 and 684 mM sucrose to determine the response of M. dubius to these concentrations of an impermeable solute. The asymptotic values reached in 684 mM sucrose show greater shrinkage than for 380 mM sucrose. Thus the

FIGURE 2.3.

The average per cent wet weight change of male and female M. dubius incubated in 190 mM NaCl, KCl and in 380 and 684 mM sucrose at 37°C. (All lines drawn by inspection).

The symbols represent:




60
50
40
30
20
10
0
−10
−20
−30
−40
PERCENT CHANGE IN WET WEIGHT
0
15
30
45
60
75
90
105
120
135
150
165
180
TIME (MINUTES)

weight response is in the expected sequence. The response in 190 mM NaCl and KCl is however an apparently greater swelling at 180 minutes than occurred in 386 mM NaCl and KCl. If the swelling in these solutions were due to passive penetration of the cations down their concentration gradient, reduction of this gradient should give a reduction in the rate of swelling. This has not occurred as far as can be judged from the values at 180 minutes. This observation coupled with the fact that no passive penetration of cations occurred at $4^{o}C$ suggests that the movement of cations is not simply controlled by concentration gradients.

2.3.4. M. dubius was incubated in a range of Na and K salts to determine the effect of anions on swelling in hyperosmotic media. M. dubius was incubated in hyperosmotic Na and K NO_3, CO_3, SO_4 and HCO_3 solutions with osmotic pressures equal to that of 342 mM NaCl and KCl. The results are shown in table 2.1. Graphs of the results showed that all the values were asymptotic at 180 minutes, except for the values for $NaHCO_3$ and $KHCO_3$.

TABLE 2.1. The mean weight changes of M. dubius in various hyperosmotic Na and K salt solutions at 37°C.

	No. Worms		Initial Reaction	Reaction at 180 mins.
NaCl	5	Female	Shrank -17%	Recovered to +13%
	2	Male	" -22%	" " +12%
KCl	3	Female	" -0.3%	" " +36%
	6	Male	" -1.2%	" " +50%
$NaNO_3$	2	Female	" -25-30%	" " -12-15%
	2	Male	" -17-20%	" " -6-12%
Na_2CO_3	2	Female	Swelling	Swelled +25-30%
	2	Male	"	" +45-50%
K_2CO_3	2	Female	"	" +25-30%
	2	Male	"	" +55-60%
Na_2SO_4	2	Female	Shrank -12-18%	Recovered to -5-7%
	2	Male	" -18-20%	" " -5-7%
K_2SO_4	2	Female	" -16%	" " -10%
	2	Male	" -16%	" " -2%
$NaHCO_3$	2	Female	" -13-18%	" " +18%
	2	Male	" -13-18%	" " +28%
$KHCO_3$	2	Female	" -13%	" " +12%
	2	Male	" -18%	" " +25%

A positive sign indicates a weight increase and a negative sign a weight loss.

$NaNO_3$, $NaHCO_3$, $KHCO_3$, Na_2SO_4 and K_2SO_4 all resulted in initial weight loss with variable weight recovery at 180 minutes. Na_2CO_3 and K_2CO_3 resulted in immediate swelling which continued to 180 minutes. Thus the anions CO_3 and HCO_3 gave similar results to NaCl in as much as the worms were swollen at 180 minutes. SO_4 and NO_3 ions cause the worm to remain shrunken at 180 minutes, having apparently stopped inward transport of Na and K ions. CO_3 and HCO_3 ions in association with K ion result in swelling at 180 minutes. SO_4 ion reduced the uptake of K, resulting in weight loss at 180 minutes. This suggests that the tegument is permeable to Cl, CO_3 and HCO_3 ions but not to SO_4 ion and is slightly permeable to NO_3 ion.

2.3.5. In order to determine whether or not the internal hydrostatic pressure of M. dubius affects the rate and degree of water uptake, the worm was incubated in 3 sucrose solutions. These were 300 mM sucrose, which has an osmotic pressure equivalent to 150 mM NaCl, 220 mM sucrose and 380 mM sucrose. Thus the response in these three solutions may be compared directly as the relative osmotic pressures are 4253 mm Hg (220 mM sucrose), 5799 mm Hg (300 mM sucrose), a difference of 1546 mm Hg, and 7346 mm Hg (380 mM sucrose), a difference of 1547 mm Hg. Thus the amount of swelling and shrinking which took place in the 220 mM and 380 mM solutions respectively was caused by the same absolute osmotic gradient. The results are shown in table 2.2.

TABLE 2.2. The mean per cent wet weight change of M. dubius males and females incubated in 220, 300 and 380 mM sucrose at 37°C.

Time	220 mM Sucrose		300 mM Sucrose		380 mM Sucrose	
(mins)	Females (2)	Males (2)	Females (2)	Males (2)	Females (1)	Males (2)
0	0	0	0	0	0	0
15	+6.6	+10.7	- 2.8	- 2.7	- 7.2	-12.9
30	+8.4	+12.7	- 4.7	- 4.8	-12.2	-15.3
45	+9.7	+13.9	- 5.8	- 6.8	-14.3	-16.7
60	+8.6	+10.7	- 7.7	- 8.8	-17.0	-19.8
75	+8.0	+ 7.8	- 8.8	-11.8	-18.4	-22.0
90	+7.9	+ 6.1	-10.4	-13.4	-19.8	-23.3
105	+6.4	+ 4.5	-11.7	-14.8	-21.3	-24.4
120	+5.2	- 0.6	-12.7	-14.7	-22.7	-25.0
150	+4.6	- 6.0	-14.8	-18.5	-25.2	-26.8
180	+2.6	- 7.8	-16.8	-20.1	-26.7	-28.1

The figure in parenthesis indicates the number of worms from which the mean is estimated. A positive sign indicates an increase in weight and a negative sign a decrease.

Both males and females lost weight in iso-osmotic (300 mM) sucrose (table 2.2). Thus the weight change of this group has been deducted from that of other two groups so that the wet weight change

from solutions with osmotic pressures 1546 mm Hg above and below the 300 mM group may be directly compared. The result is shown in figure 2.4.

This result shows that both male and female worms swell more readily than they shrink. Females swell +19% and males up to +20% in a solution with osmotic pressure 1546 mm Hg below the control group and females shrink -10% and males up to -10% in a solution with osmotic pressure 1547 mm Hg above the control group.

Females cease to change in weight once equilibrium with the solution is attained. Males however make a compensatory weight change under both conditions.

2.3.6. M. dubius was incubated in hyperosmotic solutions of $MgCl_2$ and $CaCl_2$ with osmotic pressure equivalent to 342 mM NaCl. $CaCl_2$ produced a result similar to hyperosmotic sucrose with males and females losing 22 - 26% of their weight and remaining at this level until 180 minutes after the incubation was commenced. $MgCl_2$ gave a similar result with a weight loss of 12 - 18% at 180 minutes after the incubation was commenced. These results indicate that M. dubius does not actively accumulate Ca and Mg ions, and that the tegument is not permeable to these ions.

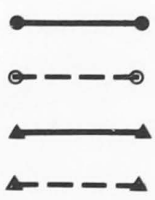
2.3.7. M. dubius was incubated in hyperosmotic solutions of LiCl, $LiNO_3$, NH_4Cl and $(NH_4)_2SO_4$ with osmotic pressures equivalent to 342 mM NaCl.

FIGURE 2.4.

The average per cent wet weight change of male and female M. dubius incubated in 220 and 380 mM sucrose at 37°C corrected for the average per cent wet weight change of the control group incubated in 300 mM sucrose.

(All lines drawn by inspection).

The symbols represent:

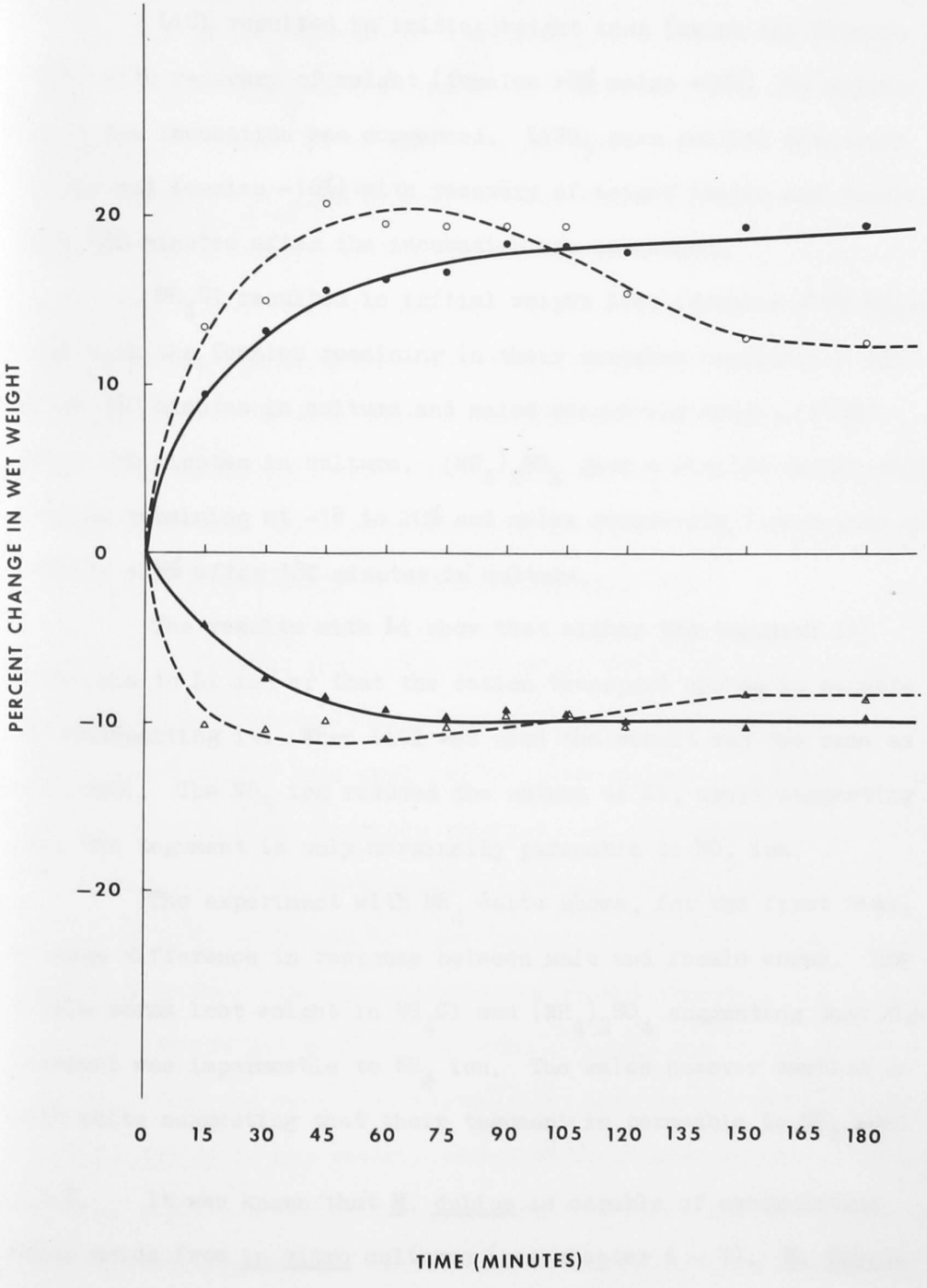


Female - 220 mM sucrose

Male - 220 mM sucrose

Female - 380 mM sucrose

Male - 380 mM sucrose


20
10
0
−10
−20
PERCENT CHANGE IN WET WEIGHT
0
15
30
45
60
75
90
105
120
135
150
165
180
TIME (MINUTES)

LiCl resulted in initial weight loss (males and females -16%) with recovery of weight (females +6% males +22%) 180 minutes after the incubation was commenced. $LiNO_3$ gave initial shrinkage (males and females -16%) with recovery of weight (males and females +2%) 180 minutes after the incubation was commenced.

NH_4Cl resulted in initial weight loss (females -18% males -2%) with the females remaining in their shrunken condition (-18%) after 180 minutes in culture and males recovering weight (+14%) after 180 minutes in culture. $(NH_4)_2SO_4$ gave a similar result with females remaining at -18 to 20% and males recovering from a loss of -10% to +25% after 180 minutes in culture.

The results with Li show that either the tegument is permeable to Li ion or that the cation transport system is capable of transporting it. When LiCl was used the result was the same as with NaCl. The NO_3 ion reduced the uptake of Li, again suggesting that the tegument is only marginally permeable to NO_3 ion.

The experiment with NH_4 salts shows, for the first time, a large difference in response between male and female worms. The female worms lost weight in NH_4Cl and $(NH_4)_2SO_4$ suggesting that the tegument was impermeable to NH_4 ion. The males however swelled in both salts suggesting that their tegument is permeable to NH_4 ion.

2.3.8. It was known that M. dubius is capable of accumulating amino acids from in vitro cultures (see chapter 6 - 7). M. dubius was incubated in hyperosmotic sucrose (with osmotic pressure

equivalent to 342 mM NaCl) containing 50, 200 and 500 mg/l L - leucine to see whether this amino acid, which is concentrated by M. dubius, affects the permeability of the bounding membrane. Hyperosmotic Na_2SO_4 containing the same concentrations of L - leucine was similarly tested. There was no difference between the responses of the worms in the sucrose solutions. Sucrose alone caused an average weight loss of 34%, sucrose + 50 mg/l leucine 39%, sucrose + 200 mg/l leucine 38% and sucrose + 500 mg/l leucine 36%. The same result was obtained with Na_2SO_4. Thus the presence of an amino acid which is normally accumulated by M. dubius did not affect the permeability of the bounding membrane.

2.3.9. A further range of hyperosmotic solutions was tested. An experiment to compare Hanks balanced salt, sucrose, and glucose solutions with the same real osmotic pressure was performed. After 180 minutes in Hanks balanced salt solution the female worms were, on average, still at their original weight ($\pm$3.0%). Sucrose resulted in 22 - 35% weight loss for males and females and glucose resulted in 20 - 24% weight loss in males and females. The weight loss in sucrose and glucose proceeded linearly and at 465 minutes the worms had not recovered any weight. This result shows either that M. dubius is not readily permeable to glucose or that glucose is very rapidly metabolised. The results further show that M. dubius is not readily permeable to sucrose, and that the worm becomes more hypotonic and loses water if replacement cations are not available.

Urea, glucose, fructose, alanine and serine were tested at concentrations to give an osmotic pressure equivalent to 342 mM NaCl. Urea resulted in initial weight loss (average -5%) with recovery (+4-6%) at 180 minutes. Glucose resulted in continued loss up to 180 minutes (average 12-16%), fructose gave the same result as glucose with a weight loss of 9-12% at 180 minutes. Alanine gave a continual weight loss to 18-20% at 180 minutes and serine a linear weight loss to 14-16% at 180 minutes. Thus either M. dubius is not readily permeable to any of these compounds or they are rapidly metabolised to an osmotically inactive state.

Leucine is insufficiently soluble in water at 37°C to give an hyperosmotic solution, so it could not be tested.

2.3.10. The results in chapter 2 show that a cation pump or pumps exists in M. dubius body wall which is capable of inward transport of Na and K ion. In Hanks balanced salt solution however there is no appreciable change in weight over 180 minutes. Thus either the cation pump(s) has ceased to operate, or an equilibrium of cation fluxes inward and outward has been established, resulting in no net change in cation content, and thus in water content and weight.

M. dubius was incubated in Hanks balanced salt solution containing NaCN. This material did not affect the weight of the worms over 180 minutes when their weight changes were compared to worms incubated in Hanks balanced salt solution containing the same amount of NaCl. Nor was the motility of the worms affected.

Similar experiments were performed in Hanks containing the cardiac glycosides Scillarin A and Ouabain (Strophanthin G) which selectively inhibit cation pumps (Glynn (1957)). Scillarin A was used at a final concentration of 1×10^{-3} and Ouabain at 2.0×10^{-3} mg/ml. The glycosides were dissolved in 70% ethanol and this solution was added to the culture. The same volume of 70% ethanol (200 μl) was added to the Hanks balanced salt solution control group. Scillarin A and Ouabain did not affect the weight of the worms used, as the groups showed average weight changes of -2.2% for Hanks balanced salt solution, +1.5% for Hanks plus Ouabain and +4.5% for Hanks plus Scillarin A at 37°C. M. dubius was incubated in Hanks balanced saline at 4°C for 180 minutes. At 180 minutes female worms had reached asymptotic swelling of +8.2% and males +3.8%. These weight changes are relatively small, but may reflect the inward diffusion of Na ion, perhaps balanced, in part, by outward diffusion of K ion.

2.4. Discussion.

It has been postulated that active transport of Na and K by muscle and nerve involves the exchange of Na for K in one to one proportions which eliminates the necessity for the movement of other ions, particularly anions to maintain electrical and osmotic equilibrium. The data presented in chapter 2 shows the existence of an active mechanism or mechanisms, capable of accumulating Na and

K against a concentration gradient and hydrostatic pressure gradient. This characteristic is demonstrated by the movement of water into the worm when it is placed in a hyperosmotic solution of these cations. Initial shrinkage followed by swelling shows that water initially leaves the worm down the osmotic gradient, and then the net flow is reversed as ions are transported into the worm. That the process is an active one is shown by the failure of this phenomonon to occur in an osmotically compensated solution at $4^{\circ}C$, when the metabolic activity of the worm would be greatly reduced or eliminated.

When M. dubius is incubated in hyperosmotic KCl, swelling results after 15 minutes. In NaCl, swelling occurs after 135-150 minutes. This suggests either that the Na pump is less efficient, or that the K pump is capable of transporting Na ion. This suggestion is further supported by the lower degree of swelling which results at 180 minutes in NaCl. Conway (1954) described a similar non-specific cation pump in yeast. Garrahan and Glynn (1967b, 1967c) described the cation pumps of red blood cells in detail. They investigated the response of the pumps in various media, using labelled cations, and concluded that the pump was capable of Na : Na exchange or Na : K exchange. The latter process occurred to the virtual exclusion of the former if K was present in the medium at a concentration of 5 mM. Thus the exchange cation pump is capable of exchanging Na for Na. It was, however, a 1:1 exchange and no net change in Na content of the red cell was possible. The mechanism

may apply to the accumulation of K by exchange for Na, by M. dubius but Na could not be accumulated to cause swelling by a 1:1 exchange pump. Thus the cation pump of M. dubius is different from that of the red cell membrane.

Garrahan and Glynn (1967c) showed that the red blood cell membrane is equipped with an exchange cation pump. The pump in M. dubius could not be an exchange pump or swelling would occur in Na_2SO_4. As swelling has not occurred, the inward movement of the Na ion must be accompanied by its anion. Thus the entry of Na and K ions from chloride solutions cannot be wholly the result of exchange pumping under the experimental conditions employed. Some weight recovery does occur over 180 minutes. This may be due to slow penetration of SO_4 ion into the worm.

Incubation in hyperosmotic LiCl resulted in swelling of both males and females. This may have been the result of Li ion replacing Na ion in the Na pump or simply due to the penetration of Li ion, which has a hydrated diameter of 5.5 Å, through the membrane.

Swelling ultimately occurred in male worms incubated in hyperosmotic NH_4Cl and $(NH_4)_2SO_4$, but female worms remained in a shrunken condition. This difference in response is the only occasion on which the responses of the two sexes of M. dubius differed. The difference in response between the sexes when incubated in $(NH_4)_2SO_4$ indicates either that NH_4 ion changes the nature of the male tegument to allow the penetration of SO_4 ion, or that NH_4 ion is assimilated by exchange with another cation so that

the accompanying anion does not need to penetrate the tegument. The second appears the more likely explanation and points to a difference in the nature of the cation pumps of male and female worms.

Czáky (1965) defined active transport as transport against a chemical and/or electro-chemical gradient. It is known from the data in chapter 3, and that presented above, that Na and K are being transported against a chemical and hydrostatic pressure gradient. Thus one of the conditions given by Czaky has been fulfilled.

The membrane potential of M. dubius depends on the distribution of all the charged particles on both sides of the membrane. Thus the membrane potential in NaCl or KCl could be, and probably is, different from the membrane potential which exists in situ. Neither of these potentials have been determined, so it is not known whether or not the observed movement of cations is up or down the electrical gradient. This question could only be resolved by applying a short circuit to the membrane and then measuring the current flow in the test media. The current flow would then represent the movement of charged ions under the influence of the cation pumps, and any concentration gradient which the system applied. No such data is available for M. dubius so only indirect evidence may be cited.

Evidence for active transport is provided by the occurrence of increased swelling when M. dubius is incubated in 190 mM NaCl and KCl. The increase in swelling occurred despite the presence of a decreased inward chemical gradient, which would result in less

passive entry of cations into the worm. Thus it must be attributed to the activity of the cation pumps which transport these ions until equilibrium is reached with membrane leakage of the ions down their concentration gradient, and against the hydrostatic pressure resulting within the worm from the inflow of water. At this point greater swelling would result in the 190 mM solutions than in the 386 mM solutions due to the lower external osmotic pressure. If, on the other hand, the entry of these cations was due to diffusion down their concentration gradients, swelling should be observed at $4^{\circ}C$ in both NaCl and KCl and the degree of swelling in 190 mM NaCl and KCl should be far less than in 386 mM NaCl and KCl. Neither of these in fact occur, so some energetic process must be involved in the accumulation of cations from hyperosmotic media.

If M. dubius maintains its volume by maintenance of a double-Donnan equilibrium with the active extrusion of Na, the cardiac glycosides should produce swelling in Hanks balanced salt solution. No notable swelling has occurred, so it must be concluded either that a double-Donnan equilibrium is not utilised, that the body wall is sufficiently semipermeable to prevent the inflow of Na, or that the cardiac glycosides have not inhibited the cation pumps.

Garrahan and Glynn (1967d) demonstrated that cation transport against a concentration gradient is accompanied by the hydrolysis of adenosine triphosphate (ATP) by an ouabain sensitive mechanism. If the hydrolysis of ATP by this mechanism was prevented cation transport ceased. Thus the failure of the cardiac glycosides

to affect the pumps of M. dubius may be due to the location of the energy supplying reactions within the body wall. Reduction in temperature to 4°C should, however, eliminate or greatly reduce the metabolic activity of M. dubius. The results show that transport of cations is also greatly reduced at 4°C. Thus it is concluded that a metabolically dependant supply of energy is necessary for the operation of the alkali cation transport system.

Incubation of M. dubius in 684 and 380 mM sucrose, which have a tonicity equivalent to 342 and 190 mM NaCl, shows that shrinkage varies directly with the tonicity of the incubation medium, suggesting that the behaviour of M. dubius in a solute which it cannot transport is that of an osmometer. The fact that weight loss does not continue at the initial rate, but becomes almost asymptotic shows that the membrane surrounding M. dubius is reasonably semipermeable, and is capable of retaining cations against a high concentration gradient. Fairbairn (1957) and Schopfer (1924, 1925) reported similar results with Ascaris and Parascaris spp., indicating that these genera are capable at least of controlling gross cation movements between their interior and their environment; in the same manner M. dubius is capable of imposing a metabolically dependent control on the movement of cations inward from the environment. Siddiqi and Lutz (1966) performed similar experiments with Fasciola gigantica and describe this animal as "highly poikilosmotic", as it loses and gains salts and water to come to equilibrium with its environment. Thus this trematode's cuticle differs from those of

Ascaris and Moniliformis. Harris and Crofton (1957) reached a similar conclusion and consider that the muscles of Ascaris act against the hydrostatic skeleton produced by the internal turgor pressure.

Fairbairn (1957) reported that Ascaris sp. does not swell in 20% sea water as much as would be expected on the basis of shrinkage of this worm in hypertonic media. Fairbairn explained this in terms of the elasticity of the cuticle producing a hydrostatic pressure within the worm. Fairbairn further reported that the cuticle of the worm will burst if the incubation medium is made too dilute. In distilled water M. dubius swells to 160% of initial weight for females and 180% for males. Rupture of the tegument of M. dubius has not been observed, but small bubbles appeared under the surface layers of the body wall. Experiments on worms in sucrose solutions show that they tend to swell more than they shrink under the influence of the same relative osmotic gradient. If the pseudocoel fluids of M. dubius were under a turgor pressure, this would be expected to balance the osmotic gradient created by a hypo-osmotic medium, and result in a smaller degree of swelling than shrinking under the same absolute osmotic gradient. This has not occurred so it is concluded that internal hydrostatic pressure does not influence the rate or extent of inflow of water with this degree of swelling. Similar results were obtained by Siddiqi and Lutz (1966) with Fasciola gigantica which gained water far more readily than it was lost.

Incubation of M. dubius in hyperosmotic solutions of $CaCl_2$ and $MgCl_2$ resulted in a sustained weight loss indicating that the body wall of M. dubius is not permeable to these ions, and that they are not actively accumulated.

Rothman and Fisher (1964) and evidence in chapters 6 - 7 show that M. dubius can accumulate amino acids from in vitro culture media. The mode of uptake of amino acids forms the second part of this thesis. One possible mode of uptake is pinocytosis. If pinocytosis is involved in the uptake of amino acids, then culture fluid would also be ingested, perhaps leading to changes in weight. If some form of membrane mediated transport were responsible, the mechanism involved may result in changes in the permeability properties of the bounding membrane. The fact that adding leucine to hypertonic media e.g. sucrose and Na_2SO_4 did not alter the response argues against any change in the permeability resulting from active transport of amino acids. If pinocytosis is the mechanism of uptake it must result in the ingestion of only small amounts of incubation medium, or else the medium ingested is rapidly eliminated.

A further range of compounds was tested for their ability to penetrate the bounding membrane of M. dubius. The worm proved to be relatively impermeable to glucose, alanine and serine. Impermeability to glucose is significant in the light of the work of Read and Rothman (1958), who showed that a source of host dietary carbohydrate was essential for the growth and reproductive activity

of M. dubius. Data will be presented later in this thesis to show that M. dubius is capable of concentrating glucose from in vitro culture media. This would suggest that no significant amount of glucose, leucine, serine or alanine, all of which the worm is capable of concentrating, is assimilated by diffusion of these materials into the worm.

The absence of consistently significant weight changes in M. dubius cultured in Hanks balanced saline, shows that this medium is isotonic with both sexes of the worm. It also indicates that either the cation pumpshave ceased to operate or that some equilibrium has been reached which maintains constant weight. The second is the more likely alternative and suggests that cation exchange pumping may be occurring. This conclusion is supported by the data to be presented in chapter 3 which shows that both sexes of M. dubius maintain high levels of K ion and low levels of Na ion in the body wall. The slower accumulation of Na ion compared to the accumulation of K ion is further evidence that Na ion is a less desirable element. This suggests that Na is being pumped out, against a concentration gradient and K is being pumped in, also against a concentration gradient. Hanks balanced salt solution contains a high concentration of Na ion, (137 mM), and a low concentration of K ion, (5 mM). Thus if M. dubius were freely permeable to both ions and were to come to Donnan equilibrium with Hanks balanced saline it would gain large amounts of Na and lose K. The result however would be that the worm would swell in the same way

as tissue cells, due to the presence of indiffusable organic metabolites within the tissues. (Tosteson (1963)) Thus if M. dubius is incubated in Hanks balanced saline with its cation pumps inactivated, the worms should swell. Slight swelling occurred in Hanks balanced saline at 4°C but none was observed in the presence of Scillaran A, Strophanthin G or NaCN. As none of the inhibitory compounds exerted any detectable effect on the body weight of M. dubius in Hanks balanced saline, it was concluded that these materials were either incapable of inhibiting this system(s) or that they were unable to reach them. The slight swelling which accompanies temperature inactivation of the cation transport system(s) in Hanks balanced salt solution suggests that the tegument of M. dubius is not readily permeable to Na or K.

The foregoing results suggest that M. dubius is equipped with an energy dependent system which is capable of accumulating Na and K ion against a concentration gradient. This system is capable of accumulating ions and water from some in vitro culture media, by inward transport of the cation and passive redistribution of the anion, rather than exchange of the transported cation for an internal cation. The balance of cations within the worm (see chapter 3) indicates however that this same system, or a seperate mechanism, is capable of exchanging Na in favour of the inward transport of K. The absence of swelling in Hanks balanced saline at 37°C, where both Na and K are present externally, also indicates that the inward cation transport system is balanced in some way by outward transport.

Conway (1954) described a similarly adaptable pump system in yeast. If Na and K were simultaneously present the yeast would accumulate K. If Na alone were present it would accumulate Na which was later discharged when K became available. Garrahan and Glynn (1967a) showed that the resealed ghosts of erythrocytes exchanged Na for Na in the absence of external K, with no accumulation of Na.

Lubin (1963) gives a long list of enzymes which require K ion as a cofactor rather than Na ion, including enzymes involved in glycolysis and the tricarboxylic acid cycle, and enzymes which are essential for the biosynthesis of small molecules, and for protein synthetis. Thus one of the functional reasons for the existence of cations pumps may be to ensure the supply of K for enzyme cofactors. K is probably also necessary to maintain nerve and muscle excitability. However, K would be freely available to M. dubius from the gut so some further significance appears to be needed to explain why this parasite is equipped with active cation pumps. These pumps may help the animal to maintain its osmotic equilibrium, but appear in fact to create an osmotic imbalance by actively accumulating both Na and K, which must be eliminated, or bound, to prevent swelling. Such mechanisms are clearly operating in Hanks balanced saline and in vivo as no evidence of swelling is seen under these circumstances. It is difficult to understand why a parasite which lacks an identifiable excretory system should accumulate cations to this extent and create a water elimination problem. It would appear that some further physiological function should be sought.

CHAPTER 3

THE INTERNAL OSMOTIC PRESSURE AND Na, K, Ca AND Mg CONTENT OF M. DUBIUS (ACANTHOCEPHALA)

3.1. Introduction.

Chapter 3 deals with the measurement of the concentrations of the cations Na, K, Ca and Mg in M. dubius, and in the contents of the rat gut in the region occupied by the worm. These results are supplemented by measurements of the osmotic pressure of tissue fluids from M. dubius and the rat gut contents, using a freezing point depression method. An account is also given of attempts to localise histochemically the distribution of Na, K and Cl ions within M. dubius.

The ability of vertebrate cells to accumulate K and maintain low concentrations of Na has been widely studied, especially in nerve and muscle cells which possess specialised mechanisms to maintain their cation distribution. A limited amount of experimental data is available for parasitic helminthes and free-living nematodes.

Myers (1966) examined the inorganic ion content of the free-living nematode Panagrellus redivivus. Myers cultured his nematodes on oatmeal plates and collected them in Fenwick's salt solution. His results are given as mg element per gram of dry weight of worm, so it is impossible to calculate the solution strength of each of the elements. He determined that Na levels in

the worm remained at only half the concentration of the salt solution in which it was maintained. K levels on the other hand, were approximately ten times the level which existed in the medium. Ca levels were approximately iso-osmotic with the medium and Mg was accumulated 100 fold. Thus it would appear that this nematode is capable of maintaining alkali metal cation gradients in the same direction as the gradients maintained by mammalian tissue cells. When P. redivivus was cultured in distilled water for 24 hours, the Na concentration in the worm fell to about one third of the previous level, but remained higher than the concentration in the medium (zero). K levels also fell to a level only 2.5 times that which was maintained in the original salt solution. Ca and Mg levels were not significantly altered by incubation in distilled water for 24 hours. Thus it would appear that P. redivivus is capable of establishing, and maintaining, Na and K ion concentration gradients.

Rogers (1945) analysed the perienteric fluid of Ascaris lumbricoides for Na and Cl and found that Na ranged from 114 to 128 mM Na and Cl from 52.6 to 59.5 mM Cl. He found little variability in his estimates but attributed this to the hosts (pigs) all being post-absorptive when slaughtered and to the results having been obtained from fluids pooled from five worms. The Cl content, mM, of Ascaris is approximately half the Na content. Rogers suggested that this difference might be accounted for by negatively charged radicals of fatty acids which neutralise the positive charge of the Na ion. Rogers did not give figures for any of these elements in the pig gut

or for the K concentration in the worm. Thus it is impossible to determine whether or not the Na concentration is in passive equilibrium with the gut fluids or in a maintained equilibrium.

Hobson, Stephenson and Eden (1952) examined the inorganic ion content of the pig gut and Ascaris lumbricoides body fluid, and determined that the concentration of cations in the body fluid of Ascaris is essentially the same as in the worm's environment. These workers exposed Ascaris to various media and recorded the changes in inorganic ion composition. The Na concentration within the worms varied only slightly over a range of external concentrations. K and PO_4 concentrations fell regardless of the K concentration of the medium used. Ca and Mg levels remained essentially unaltered.

Schopfer (1925) estimated the osmotic pressure of the inorganic ion component of Ascaris body fluids and the pig gut fluids. He estimated the cation osmotic pressure of the worm body fluids to be 1.81 atm (equivalent to 35.6 mM NaCl) hypo-osmotic to the gut fluids. Hobson, Stephenson and Beadle (1952) estimated the osmotic pressure of Ascaris body fluid to be 65 mM NaCl hypo-osmotic to the gut fluids for the total osmotic pressure and 32 mM for the cation osmotic pressure. This is in close agreement with the 35.6 mM difference estimated by Schopfer (1925). Fairbairn (1957) comments that all osmotic pressure measurements of body fluids may be reduced by the volatilisation of volatile fatty acids. Schopfer did not offer any reason for the existence of this osmotic pressure difference, but commented that some factor other than osmosis must

affect the movements of water between the worm and its environment.

The evidence suggests that the inorganic ion content of Ascaris in its normal environment is the result of passive diffusion. The worm is however, capable of maintaining its cation content in vitro. The body fluid of Ascaris is hypo-osmotic to its environment, and as the cuticle of this worm is permeable to water, (Fairbairn (1957)) the worm must have some mechanism for conserving water and resisting the movement of solutes. Harris and Crofton (1957) showed that a high turgor pressure is maintained within Ascaris, so the mechanism of fluid conservation must be of high efficiency.

Goodchild, Dennis and Moore (1962) examined the Ca, Mg, K and Na content of the anterior, middle and posterior thirds of Hymenolepis diminuta (Cestoda). They gave their results as the weight of element per 100 units dry weight of worm. They showed that the concentration of K exceeded that of Na by factors of 3.44, 2.73 and 2.23 for the anterior, middle and posterior regions of the worm respectively. The order of abundance of the cations is K, Na, Ca and Mg. Goodchild, et al, did not give any figures for the environmental fluid of the tapeworm so the significance of these levels in relation to the environment cannot be judged. The relative abundance of Na and K is the same as that for mammalian cells.

von Brand (1939) detected the presence of the cations Na, K, Mg, Ca and Fe spectrographically in the acanthocephalan Macracanthorhynchus hirudinaceus. He also detected the anions PO_4,

SO_4, CO_3 and Cl. He did not determine the concentration of these materials, and comments that they deserve no discussion as they are widely distributed in nature. Later von Brand and Saurwein (1942) made further spectrographic analyses of M. hirudinaceus and detected Na, Ca, Mg, Mn, Al, Fe, Cu, K, P and Si. They estimated Cu and Fe quantitatively because of the importance of these metals in respiratory pigment but were not concerned with the osmotic characteristics of the worm. The amounts of Fe and Cu were found to be 0.30% and 0.01% respectively of the weight of ash from dried female worms.

It can be seen that there is little published data on inorganic ion content of the Acanthocephala. An analysis of the worm Moniliformis dubius was undertaken to determine the distribution of cations in the worm tissues and, if possible, to gain an insight into the osmotic relationships of the worm in its environment. Spectrographic and histochemical methods were used for determination and localisation of the cations. A freezing point depression method was used for osmotic pressure determinations.

3.2. Materials and Methods.

3.2.1. Spectrographic methods.

Fresh worms were removed from the gut of rats killed by a blow on the head. The worms were wiped between the fingers to remove faecal material, and were then weighed on a Mettler automatic

balance. Two ligatures of surgical thread were tied around the worm as close as possible to the centre, and the worm was cut between the ligatures. The two halves were weighed. The end was cut off one of the halves and the pseudocoel fluid squeezed out into a tared beaker. This fluid was weighed. The remaining body wall was cut longitudinally, blotted dry on a piece of absorbent tissue, and weighed in a tared beaker. Thus the wet weights of the components to be analysed were known. To determine whether or not the two halves of the worm contained different amounts of cations the anterior end and posterior end of successive worms were alternately assayed whole and subdivided. This process permits statistical analysis to determine differences in total content and differences in distribution between the body wall and pseudocoel. Male worms were treated whole by the same methods. The components of the worms were dried for twelve hours in an oven at 105°C and weighed. They were then digested with 0.5 ml of 2N - HNO_3 plus 0.5 ml of 30% perchloric acid, by gentle heating on a hot plate until a clear dull yellow solution resulted, which was then evaporated to dryness. The charred material which remained was soaked in 2N - HNO_3 for two hours at room temperature. It was then poured into a No. 2 glass sinter funnel containing a small volume of double glass distilled water, and was filtered by gravity into a volumetric flask. The digestion vessel was extensively rinsed with double glass distilled water and the rinsings filtered. The digest arising from the pseudocoel was diluted volumetrically to 100 ml, after soaking in 2 mls of

2N - HNO_3, the body wall and males to 50 ml after soaking in 1.0 ml of 2N - HNO_3 and the whole worm extract to 250 ml after soaking in 5 mls of 2N - HNO_3. These volumes were used because they produced concentrations of cations suitable for the analytic instrument used. All Na and K unknowns contained 800 ppm (parts per million) Li, achieved by adding 20 ml, 10 ml and 50 ml respectively of a 4,000 ppm $LiNO_3$ solution.

Standard solutions of the cations under consideration were prepared from 800 ppm solutions of the analytical grade reagents Na_2SO_4, K_2SO_4, $Mg(NO_3)_2$ and $Ca(NO_3)_2$. The standards used were Na, 1, 2, 3 and 5 ppm; K, 1, 2, 3, 4, 7, 10, 15, 20, 25, 30 and 40 ppm; Ca 0.25, 0.50, 1.0 and 1.5 ppm, and Mg, 1, 2, 3, 4 and 7 ppm. These standards were prepared from the 800 ppm solutions by serial dilution with double glass distilled water, which was then used as a blank for calibration of the instrument. The Na and K standards also contained 800 ppm $LiNO_3$.

The efficiency of recovery of Na and K from the above process was determined by assaying a known volume of volumetrically prepared Na_2SO_4 and K_2SO_4 solution. The results were 90.52% for Na and 96.12% for K. These corrections have been applied to the experimental results.

The pseudocoel fluid and eggs of M. dubius were also analysed. Rat gut fluid isolated from ingesta surrounding the worms in 5 rats was assayed in the same way. Five female Macracanthorhynchus hirudinaceus were analysed in the same way as M.

dubius females. To determine the ability of M. dubius to control its cation content, worms were analysed after the following treatments (1) swelling in 1.0, 1.5 and 2.0% sucrose solutions for 2 hours; (2) culturing for 2 days in Hanks balanced salt solution and the tissue culture medium Parker - 199.

The instrument used for cation analysis was a Perkin - Elmer Atomic Absorption spectrophotometer, Model 303. This instrument was used with hollow cathode K, Ca and Mg spectral emission lamps. The Na lamp used was a spectral vapour lamp. The flame of the instrument was operated 8 psi (pounds per square inch) acetylene and 26 psi compressed air. The instrument was operated at the emission wavelength of the element concerned, viz:- for Na, 5890 Å; K, 7665 Å; Mg, 2852 Å and Ca, 4227 Å.

The Na and K standards contained 800 ppm $LiNO_3$ because they were originally prepared for use with a Perkin - Elmer flame photometer, which uses a uniform concentration of Li as an internal null standard. The flame photometer was not used because the atomic absorption spectrophotometer gives greater accuracy and sensitivity. The same Na and K standards were used however, so $LiNO_3$ was added to all unknown solutions so that the solutions were as similar as possible to the standards, except for the cation being assayed.

Ca and Mg standards were prepared from analytical grade $Ca(NO_3)_2$ and $Mg(NO_3)_2$, to which 3,000 ppm strontium chloride was added to supress interference by PO_4 and SO_4 (David (1960)).

The instrument was set for maximum cation sensitivity by

adjustment of the operating wavelength and was then set at zero absorbance using double glass distilled water from the same still as the water used to prepare the standards and unknown solutions. Three sets of standard readings were taken for each of ten unknown solutions tested and all readings were replicated.

All glassware used in the preparation of the unknown solutions was cleaned by the following procedure. First it was washed in hot water and detergent, and then rinsed three times in hot tap water, and twice in glass distilled water. This was followed by soaking in dilute chromic acid. The glassware was then rinsed in three changes of glass distilled water followed by two rinses in double glass distilled water. The vessels were dried upside down, to allow maximum drainage. All glassware was stored in plastic bags between uses to prevent dust contamination. Before the glassware was used the necks were rinsed with double glass distilled water to remove any contaminants which may have accumulated on the lip during drying. The No.2 glass sinter was similarly treated between uses, except that it was never washed in detergent.

The Parker 199 tissue culture medium used in this chapter was purchased from The Commonwealth Serum Laboratories and prepared according to their recommendations.

3.2.2. The Method of Estimating Freezing Point Depression.

Freezing point depression estimates were obtained using a modified form of the apparatus originally developed by Jones (1941).

Standard solutions of NaCl containing 500, 400, 300, 200, 100 and 0 mM NaCl were volumetrically prepared. The standard and unknown solutions were sealed in separate 10 μl glass tubes and frozen in a cold alcohol bath. The frozen tubes were placed between crossed polaroids in a cold alcohol bath under a stereoscopic microscope. The ice crystals can be clearly seen in this position. The alcohol bath was allowed to warm up slowly with constant agitation to maintain the most uniform possible temperature. Zero time was taken when the last ice crystal in the most concentrated standard tube melted. The time delay for the other tubes to melt was recorded and the concentration of the unknown tube determined by interpolation on a graph of time taken to melt against concentration. All estimates were replicated if the unknown tube would withstand a second freezing.

3.2.3. Histochemical Methods.

Komnick (1962) and Kaye et al (1966) described a histochemical method for the localisation of Na in tissues. The Na was precipitated during fixation by soaking the tissue in 1% osmium tetroxide, OsO_4, containing 2% potassium antimony hydroxide, $K(Sb(OH)_6)$, at 1°C. The fixed tissues were then prepared for examination with the electron microscope. A histochemical method for the localisation of K was described by Pearse (1961), in which tissues were soaked in 12% sodium cobaltinitrite, $NaCoNO_2$, at 0°C.

Both these methods have been modified for the present work.

Na was precipitated by soaking small blocks of fresh tissue in 2% $K(Sb(OH)_6)$ at 1°C for 2 hours. The blocks of tissue were then embedded in 5% gelatine and 8μ sections were cut on a freezing microtome. The sections were rapidly dried on the slides at 45°C, and were then washed in distilled water at 1°C, dehydrated and fixed in absolute ethanol for 10 minutes, cleared in xylol, and mounted in Canada Balsam.

K was precipitated by soaking blocks of fresh tissue in 6% $NaCoNO_2$ for 2 hours at 1°C. The above procedure was used to cut and mount the sections. Cl was localised by a modification of the method of Komnick (1962). Small pieces of fresh tissue were soaked in 1% silver nitrate, $AgNO_3$, for 2 hours at 1°C. The sections were prepared in the same way as the K sections.

Control sections were prepared in all cases by soaking small pieces of unfixed tissue in distilled water at room temperature for 4 hours and then treating by the methods described above.

3.2.4. Calculation Methods.

The atomic absorption spectrophotometer gives a numerical value which is known as percentage absorption. This is an estimate of the emission wavelength absorption in a flame. Optical density is related to absorption by the function $d = \log \left(\frac{100}{100-a}\right)$ where d is the optical density and a is the percentage absorption (David (1960)). The optical density may be determined from the "absorbance/absorption" tables supplied with the instrument. Thus a graph of

concentration against optical density or absorbance may be constructed from the standard readings, and the concentration of the unknown determined from the graph. Typical graphs may be found in appendix figures 3.1 and 3.2.

The concentration of the unknown is converted into g element/g H_2O by the equation:

$$\text{g element/g } H_2O = \frac{\text{ppm x dilution vol x } 10^{-6}}{\text{wt of water in the original sample}}$$

3.3. Results.

3.3.1. Tables 3.1, 3.2 and 3.4 show the Na and K content of male and female M. dubius, female M. hirudinaceus, and the gut fluid of 5 infected rats. The Na and K content of the body wall is inclusive of the content of the body musculature. The figures in tables 3.1, 3.2 and 3.4 have not been corrected for efficiency of recovery because they have been used for statistical analysis of the data and comparison with other estimates. The figures have been corrected for efficiency of recovery in calculating the concentration of the solutions in mM Na and K in table 3.7. The methods of calculation are described in section 3.2.4. and are shown in detail in appendix 3.1.

Analysis of variance of the results for M. dubius gave the differences shown in table 3.3. The Na data for M. dubius showed significant differences between the content of the female pseudocoel of different age groups, ($p = 0.010$) and the female body wall content

TABLE 3.1. The Na and K content of the anterior and posterior ends of M. dubius female pseudocoel and body wall, whole male and female, and the rat gut fluids.

Worm Component	No. of Estimates	Ranges g element/g H_2O		Mean and Standard Deviation g element/g H_2O	
		Na	K	Na	K
Female P/coel (Ant)	9	1.17 - 2.02	0.71 - 2.09	1.53 ± 0.33	1.37 ± 0.44
" P/coel (Post)	9	1.10 - 2.54	1.05 - 2.66	1.48 ± 0.45	1.92 ± 0.52
" B.W. (Ant)	9	0.48 - 0.80	4.19 - 7.19	0.59 ± 0.11	5.94 ± 0.92
" B.W. (Post)	9	0.40 - 0.88	5.17 - 7.08	0.57 ± 0.16	5.99 ± 0.66
" Whole (Ant)	9	0.61 - 1.03	2.95 - 4.43	0.80 ± 0.14	3.71 ± 0.44
" Whole (Post)	9	0.56 - 0.91	3.26 - 5.57	0.73 ± 0.12	4.26 ± 0.82
Male Whole	3	0.91 - 1.55	5.89 - 11.40	1.34 ± 0.33	8.44 ± 2.78
Rat gut fluids	5	2.09 - 2.74	0.72 - 1.44	2.32 ± 0.26	1.04 ± 0.26

All results in this table x 10^{-3}

TABLE 3.2. The Na and K content of 16, 18 and 20 week old female M. dubius pseudocoel and body wall and whole males and females.

Worm Component	Age (weeks)	No. of Estimates	Ranges g element/g H_2O		Mean and Standard Deviation g element/g H_2O	
			Na	K	Na	K
Female P/coel	20	7	1.27 - 1.79	1.29 - 2.66	1.57 ± 0.19	2.02 ± 0.53
" "	18	7	1.10 - 1.42	0.71 - 2.10	1.25 ± 0.13	1.39 ± 0.52
" "	16	4	1.28 - 2.54	1.12 - 1.69	1.96 ± 0.52	1.43 ± 0.26
Female B.W.	20	7	0.48 - 0.88	5.20 - 6.16	0.60 ± 0.15	5.85 ± 0.33
" "	18	7	0.53 - 0.80	5.66 - 7.19	0.62 ± 0.09	6.58 ± 0.67
" "	16	4	0.40 - 0.60	4.19 - 5.64	0.47 ± 0.09	5.09 ± 0.63
Whole Female	20	7	0.57 - 1.03	3.26 - 4.55	0.76 ± 0.17	3.67 ± 0.43
" "	18	7	0.56 - 0.97	3.77 - 5.57	0.78 ± 0.14	4.45 ± 0.76
" "	16	4	0.73 - 0.81	2.95 - 4.18	0.76 ± 0.04	3.52 ± 0.62
Whole Male	21	3	0.91 - 1.55	5.89 -11.40	1.34 ± 0.33	8.44 ± 2.78

All results in this table x 10^{-3}

TABLE 3.3. The distribution of significant differences ($p = 0.050$) between the Na and the K content of various components of M. dubius.

Na (g Na/g H_2O)

Differences between	Female P/coel	Female B.W.	Whole Female	Whole Male
Ages	**	N.S.	N.S.	-
Rats	N.S.	**	N.S.	-
Ends	N.S.	N.S.	N.S.	-
Whole female	-	-	-	N.S.

K (g K/g H_2O)

Differences between	Female P/coel	Female B.W.	Whole Female	Whole Male
Ages	N.S.	**	*	-
Rats	N.S.	*	N.S.	-
Ends	*	N.S.	N.S.	-
Whole female	-	-	-	N.S.

TABLE 3.4. The Na and K content of the anterior and posterior ends of *M. hirudinaceus* female pseudocoel and body wall and whole females.

Worm Component	No. of Estimates	Ranges g element/g H_2O		Mean and Standard Deviation g element/g H_2O	
		Na	K	Na	K
Female P/coel (Ant)	2	1.34 - 1.65	1.91 - 2.13	1.49 ± 0.22	2.02 ± 0.16
" " (Post)	3	1.30 - 1.52	1.82 - 2.73	1.38 ± 0.12	2.33 ± 0.47
Female B.W. (Ant)	2	0.41 - 0.85	5.09 - 6.25	0.63 ± 0.31	5.67 ± 0.82
" " (Post)	3	0.54 - 0.75	5.56 - 6.87	0.61 ± 0.12	6.30 ± 0.68
Whole Female (Ant)	3	0.84 - 1.15	3.75 - 4.68	1.00 ± 0.16	4.28 ± 0.48
" " (Post)	2	0.89 - 1.19	3.78 - 4.32	1.04 ± 0.21	4.05 ± 0.38

All results in this table x 10^{-3}

of worms from different rats ($p = 0.010$). The female pseudocoel Na content from 16 week old worms (1.96×10^{-3} g Na/g H_2O) was significantly greater than the content at 20 weeks (1.57×10^{-3} g Na/g H_2O) which was in turn significantly greater than the content at 18 weeks (1.25×10^{-3} g Na/g H_2O).

The mean Na content of female body wall of worms removed from each of the 7 rats was calculated and is shown ($\times 10^{-3}$) with a series of lines drawn below the numbers. Each of these lines is drawn to show how groups of means differ ($p = 0.050$).

0.88 0.66 0.62 0.60 0.60 0.49 0.42

The least significant difference between the means is 0.06×10^{-3} g Na/g H_2O. Thus all the means differ significantly from 0.42 g Na/g H_2O. All the remaining means differ significantly from 0.49 g Na/g H_2O. Only two means differ significantly from 0.62, 0.60 and 0.60 g Na/g H_2O and these three do not differ significantly. 0.88 and 0.66 g Na/g H_2O are significantly different, (i.e. those means underlined differ significantly, $p = 0.050$, from the remaining means in that row).

Analysis of the K data showed significant differences between, (a) female body wall content for the age of the worms, and the rat of origin, (b) whole female content between age groups and (c) the anterior and posterior female pseudocoel content. The distribution of differences between age groups for body wall K

was

18 weeks	20 weeks	16 weeks
6.58×10^{-3}	5.85×10^{-3}	5.09×10^{-3}

The least significant difference was 0.67×10^{-3} g K/g H_2O.

The distribution of significant differences ($p = 0.050$) between female body wall K for rat of origin was ($\times 10^{-3}$)

6.92, 6.12, 6.11, 5.90, 5.71, 5.64, 4.91

The least significant difference was 0.37×10^{-3} g K/g H_2O.

The whole female K content differed significantly ($p = 0.050$) between age groups of worms. The distribution of significant differences was:-

18 weeks	20 weeks	16 weeks
4.45×10^{-3}	3.67×10^{-3}	3.52×10^{-3}

The least significant difference is 0.75×10^{-3} g K/g H_2O.

Female pseudocoel K content showed a significant difference ($p = 0.050$) between the ends of the worm. The K content of the posterior end (1.92×10^{-3} g K/g H_2O) was significantly greater than that of the anterior end (1.37×10^{-3} g K/g H_2O).

No other signficant differences ($p = 0.050$) were found in the data from *M. dubius*.

The difference in pseudocoel K content between the

anterior and posterior end was unexpected because the pseudocoel fluids are extremely mobile and it is difficult to understand how K could be concentrated in one end of this compartment. Analysis of the eggs showed (see section 3.3.2) the K content to be quite high. It is possible that the distribution of eggs may have affected the result as there may be local concentrations of eggs in the region of the uterine bell and the uterus which would increase the K content of the posterior end of the worms.

That the rat of origin and age of the worm may affect the Na and K levels of the female body wall is not surprising. It was not possible however to further investigate these differences. Additional data would have been required, for example the time of day the rat was killed, the sex of the rat, the number and sex of worms in the gut and the age of the worms. All this data was not recorded during the above experiments because its significance was not realised until the results had been analysed. The work was not repeated because the reasons for the differences observed may be expected to be complex and involve interactions in the entire ecosystem. Also the author was more interested in the physiological functions of Na and K in the worm than the fine details of the overall cation balance of the ecosystem. As all this data was unavailable care was taken to select rats of the same sex, with, as far as possible, worms of the same age and infection density for any comparative experiments on Na and K.

The results obtained from the analysis of

Macracanthorhynchus hirudinaceus females were only compared between ends of the worm, as the age of the worms and their distribution in individual pigs was not known. No significant differences were detected between the worms for body wall, pseudocoel or whole worms, or between anterior and posterior halves of the worms, for Na or K levels.

A comparison can be made of the distribution of Na and K in M. dubius and M. hirudinaceus as g Na or K/ g worm water by "t" test. The analysis showed no significant differences between the species for body wall Na or K concentrations. Comparison of the whole worms showed no significant differences for K and a significant difference ($p = 0.010$) for Na. The concentrations are 0.769×10^{-3} g Na/g worm water for M. dubius and 1.012×10^{-3} g Na/g worm water for M. hirudinaceus. The results show that the Na and K content of the two species is remarkably similar.

3.3.2. In order to determine the distribution of Na and K between the pseudocoel fluid and the eggs a pooled sample of M. dubius pseudocoel fluid was taken from five worms and analysed. The eggs were removed by centrifugation at 500 rpm (revolutions per minute) for two minutes. The supernatant was removed and the eggs rinsed in 0.25 M sucrose. The eggs were removed by centrifugation and then dried and weighed. The results are expressed as g element/g dry weight because the original weight of water in the eggs may have been altered by washing in sucrose. This enabled an estimate

to be made of the distribution of Na and K between the eggs and pseudocoel fluid. The content of the pseudocoel fluid and the eggs (g Na or g K/g H_2O) was calculated from the distribution. This result has been used in table 3.7 to estimate the mM cation concentrations in the various components of the worm. The analysis of the pseudocoel fluid given in section 3.3.1 is for whole fluid, i.e. containing the eggs. The results of the pseudocoel fluid analysis are shown in table 3.5. Addition of the results for Na and K in pseudocoel fluid and eggs gives the total concentration of Na and K in whole pseudocoel fluid, from which the percentage contribution of each was determined. The results of the calculations are also shown in table 3.5.

TABLE 3.5. The Na and K content of the pseudocoel fluid of M. dubius and the distribution of these ions between the eggs and fluid.

Component	g Na/g dry weight	Percentage contribution	g K/g dry weight	Percentage contribution
Fluid	0.01720	91%	0.01180	62%
Eggs	0.00167	9%	0.00717	38%
Total	0.01887	100%	0.01897	100%

If the results for whole pseudocoel analysis are subdivided in the proportions shown in table 3.5, and the concentrations in pseudocoel and body wall are estimated as percentage contributions, the distribution of Na and K in body wall, pseudocoel

fluid and eggs can be calculated. The result is shown in table 3.6.

TABLE 3.6. The percentage distribution of Na and K in pseudocoel, body wall and eggs of M. dubius.

	Na	K
Eggs	7%	8%
P/coel	66%	13%
Body Wall	27%	79%

All the above data (section 3.3.1 and 3.3.2) has been corrected for recovery efficiency and estimated as mM Na and K solutions (Table 3.7). The real osmotic pressure each of these solutions would exert as chlorides is also shown in the table. The real osmotic pressures were estimated from the formula given by Diem (1962).

The concentrations of Na and K (mM) shown in table 3.7 are shown in figure 3.1. The results show that whole female M. dubius is iso-osmotic with the rat gut while the whole males are hyperosmotic. The results of analysis of female pseudocoel and body wall separately show that the pseudocoel is hypo-osmotic and that the body wall is hyperosmotic to the gut. These results represent only the contribution of the cations of the worms and not the total osmotic pressure.

Data to be presented in chapter 4 shows that in Hanks balanced saline the following proportions of Na and K are non-exchangeable or exchange only slowly: female body wall Na 74%,

TABLE 3.7. The Na and K concentration of M. dubius, its components and the rat gut together with the real osmotic pressure these solutions would exert as chlorides.

	Na Concentration			K Concentration			
	g Na/g H_2O	mM Na	Real osmotic pressure (mm Hg)	g K/g H_2O	mM K	Real osmotic pressure (mm Hg)	Total real osmotic pressure (mm Hg)
Female P/coel	16.9461×10^{-4}	73.67	2678	16.9881×10^{-4}	43.56	1618	4296
Female P/coel*	15.4209×10^{-4}	67.05	2442	10.5326×10^{-4}	27.01	1000	3442
Female B.W.	6.4183×10^{-4}	27.91	1033	62.0539×10^{-4}	159.11	5700	6733
Whole Female	8.4951×10^{-4}	36.93	1339	41.2479×10^{-4}	105.70	3824	5163
Whole Male	14.8029×10^{-4}	64.36	2344	87.8013×10^{-4}	225.13	7999	10343
Rat gut	25.5186×10^{-4}	110.95	3975	10.8191×10^{-4}	27.73	1027	5002

female body wall K 24%, female pseudocoel Na 91%, female pseudocoel K 92%, male Na 21% and male K 63%. If non-exchangeable means osmotically inactive, the osmotic pressure contribution of the cations would be reduced to the levels shown in table 3.8. The figures represent the real osmotic pressure of the exchangeable cations.

TABLE 3.8. The contribution of exchangeable Na and K, as chlorides, to the real osmotic pressure of M. dubius and the rat gut.

Component	Na	K	Total
P/coel. Female	223	135	358
B.W. Female	282	4670	4952
Whole Male	1851	3221	5072
Rat gut	3975	1027	5002

Whole female worms have not been included in table 3.8 because the proportion of whole female cations which is exchangeable is not known.

Table 3.8 shows that the female body wall and whole male exchangeable cation osmotic pressures approximate the osmotic pressure of the gut. The data in chapter 4 shows a slow increase in the proportion of Na and K exchanged by the pseudocoel with Hanks balanced salt solution. Thus it appears that the cations in this region are not in fact bound, and osmotically inactive, but simply exchange very slowly under the conditions used.

3.3.3. In order to determine the proportion of cations which were retained under extreme conditions, M. dubius was incubated in 1.0, 1.5 and 2.0% sucrose at 37°C for two hours and subjected to alkali cation analysis. The results are shown in table 3.9 which also compares these results ("t" test) with the Na and K content of worms taken from the gut. Only the body wall Na had not changed significantly.

The results give an appreciation of the ability of M. dubius to retain some cations against an extreme concentration gradient. The results given above are calculated on the basis of the water content of the swollen worms. A correction was not made for swelling because it represents the real position within the worms. The worms swelled 89, 69 and 66% in 1.0, 1.5 and 2.0% sucrose respectively. The concentration of Na and K in the worms was calculated as mM Na and K and is shown in figure 3.1.

The Na and K pools which remained within the worms must be in some way distinct from that which was lost. The existence of non-exchangeable or slowly exchangeable Na and K pools was mentioned in section 3.3.2. These estimates are compared to the proportion of Na and K which was retained in sucrose.

TABLE 3.9. Comparison of the mean Na and K content ($\pm$ 1 standard deviation) of female *M. dubius* incubated for two hours in hypo-osmotic sucrose solution with that of worms from the gut. (g Na or K/g H_2O x 10^{-3})

Worm Component		Worm content after incubation in 1.0% sucrose	1.5% sucrose	2.0% sucrose	Mean content of worms from sucrose solutions	Mean content of worms from the rat gut	Difference+	Sig.	Percentage Lost	Retained
Female P/coel	(Na	0.52	0.70	0.73	0.65 ± 0.11	1.53	- 0.88	*	57.9	42.1
	(K	0.81	0.10	0.98	0.93 ± 0.11	1.63	- 0.70	*	42.9	57.1
Female B.W.	(Na	0.38	0.57	0.75	0.57 ± 0.19	0.58	- 0.01	NS	2.2	98.9
	(K	1.27	1.69	1.51	1.49 ± 0.22	5.97	- 4.48	***	75.0	25.0
Whole Female	(Na	0.40	0.58	0.44	0.47 ± 0.10	0.77	- 0.30	*	38.4	61.6
	(K	1.05	1.21	1.16	1.14 ± 0.11	3.97	- 2.83	***	71.3	28.7

+ A negative difference indicates that the content of the test worm is less than the content of worms taken from the rat gut.

TABLE 3.10. The proportion of total Na and K which is not exchanged in Hanks balanced saline over 4 hours compared to the proportion retained in hypo-osmotic sucrose after 2 hours.

	Non-exchangeable Na		Non-exchangeable K	
Component	Hanks	Hypo-osmotic Sucrose	Hanks	Hypo-osmotic Sucrose
Female P/coel	91	42	92	57
Female B.W.	74	99	24	25

Thus, the body wall has retained more cations than would have been predicted from the proportion of cations exchanged in Hanks balanced salt solution and the pseudocoel has retained considerably less.

3.3.4. Little success has been achieved in *in vitro* cultivation of Acanthocephala. In order to determine the nature of the cation balance *in vitro*, female worms were incubated for two days in Hanks balanced salt solution (1 worm) and the tissue culture medium Parker 199 (1 worm), and these worms were analysed for Na and K. The results are shown in table 3.11. The results have been corrected for efficiency of recovery.

The concentration of Na and K (mM) in the worms after 2 days incubation is shown in figure 3.1.

At the end of incubation both worms were active and continuing to shed mature acanthors into the medium. Both worms

FIGURE 3.1.

The Na and K concentration (mM or mEq/l) in the body wall and pseudocoel of female M. dubius and whole males, taken from the rat gut, Hanks balanced salt solution and after swelling in hypo-osmotic sucrose.

PSEUDOCOEL
BODY WALL
WHOLE WORM
K CONCENTRATION (mM or mEq)
200
180
160
140
120
100
80
60
40
20
0
Na CONCENTRATION (mM or mEq)
80
60
40
20
0
Ex GUT
Ex SUCROSE
Ex HANK'S SALINE
Ex GUT
Ex SUCROSE
Ex HANK'S SALINE
Ex GUT
Ex SUCROSE
Ex HANK'S SALINE

lost some weight in the culture, viz 14% and 6% respectively, some of which may be attributed to the acanthors shed. The results from these worms were compared ("t" test) with the results from female worms taken from the rat gut. The analysis showed that the only significant difference was the Na concentration of the body wall, 0.642×10^{-3} g Na/g worm water or 28 mM for worms taken from the rat gut and 1.38×10^{-3} g Na/g worm water or 60 mM for worms taken from the incubation medium i.e. the body wall Na concentration had increased 2-fold. This may indicate that the worm has reached a new equilibrium with the higher Na concentration of the medium, (111 mM Na in the gut and 137 mM in the medium) or that its cation control mechanisms are beginning to degenerate and inflow of Na ion has resulted.

3.3.5. Ca and Mg levels were estimated in female M. dubius. No analyses of male worms was performed. The results, shown in tables 3.12 and 3.13, were subjected to analysis of variance to detect significant differences between ages, rat of origin and the end of the worm analysed. Analysis of the Mg data showed no significant differences at the 5% level of probability. Analysis of the Ca data showed a significant difference between the content of the body wall from different rats. This difference arose from one worm which originated by itself from one rat. The worms from the other 4 rats did not differ signficantly.

The results in tables 3.12 and 3.13 have been calculated

TABLE 3.11. Comparison of the mean Na and K contents of female M. dubius incubated for 2 days in Hanks balanced salt solution (1 worm) and Parker 199 (1 worm) at 37°C.

Worm or component	Content of female M. dubius incubated in: Hanks balanced saline (g element/g $H_2O \times 10^{-3}$)	Parker 199 (g element/g $H_2O \times 10^{-3}$)	Mean Content (g element/g $H_2O \times 10^{-3}$)	(mM)	Worm from the rat gut + (mM)
Female P/coel (Na	1.57	1.46	1.52	66.2	73.7
Female P/coel (K	2.68	2.25	2.46	63.2	43.6
Female B.W. (Na	1.32	1.45	1.38	60.0	27.9
Female B.W. (K	7.94	6.87	7.41	189.9	159.1
Whole Female (Na	1.16	1.07	1.12	48.5	36.9
Whole Female (K	3.73	2.98	3.36	86.2	105.7

+ data from table 3.7 for comparison.

TABLE 3.12. The Ca and Mg contents (g element/g H_2O x 10^{-3}) of the anterior and posterior halves of M. dubius female pseudocoel, body wall and whole females.

Worm or Component	No. of Estimates	Range of Observations		Mean and Standard Deviation	
		Ca	Mg	Ca	Mg
Female P/coel (ant)	5	0.34 - 1.22	0.37 - 0.82	0.62 ± 0.38	0.53 ± 0.18
" " (post)	5	0.37 - 1.18	0.41 - 0.70	0.76 ± 0.32	0.59 ± 0.14
Female B.W. (ant)	5	0.14 - 1.44	0.44 - 0.65	0.42 ± 0.58	0.53 ± 0.08
" " (post)	5	0.14 - 0.67	0.44 - 0.63	0.35 ± 0.21	0.53 ± 0.09
Whole Female (ant)	6	0.24 - 0.66	0.28 - 0.73	0.38 ± 0.17	0.39 ± 0.17
" " (post)	5	0.24 - 1.27	0.32 - 0.42	0.67 ± 0.43	0.38 ± 0.04

TABLE 3.13. The Ca and Mg content (g element/g H_2O x 10^{-3}) of 21, 14 and 10 week old *M. dubius* female body wall and pseudocoel and whole females.

Worm or component	Age (weeks)	No. of estimates	Range of Observations		Mean and Standard Deviation	
			Ca	Mg	Ca	Mg
Female P/coel	21	1	1.18	0.67	1.18	0.67
" "	14	5	0.34 - 1.22	0.37 - 0.82	0.63 ± 0.39	0.56 ± 0.19
" "	10	5	0.40 - 0.85	0.40 - 0.68	0.64 ± 0.21	0.52 ± 0.14
Female B.W.	21	1	-	0.44	-	0.44
" "	14	5	0.16 - 1.44	0.45 - 0.65	0.48 ± 0.54	0.55 ± 0.09
" "	10	5	0.14 - 0.67	0.44 - 0.62	0.28 ± 0.23	0.52 ± 0.10
Whole Female	21	1	0.41	0.30	0.41	0.30
" "	14	5	0.26 - 1.27	0.31 - 0.73	0.74 ± 0.37	0.45 ± 0.17
" "	10	5	0.24 - 0.48	0.28 - 0.40	0.31 ± 0.21	0.35 ± 0.06

as mM solutions of Ca and Mg and the osmotic pressures exerted by these solutions as dissociated chlorides (Table 3.14).

TABLE 3.14. The concentration of Ca and Mg in M. dubius females and the real osmotic pressures of these solutions as chlorides.

Component	Ca		Mg		
	Conc. mM	Real osmotic pressure (mm Hg)	Conc. mM	Real osmotic pressure (mm Hg)	Total real osmotic pressure (mm Hg)
P/coel	8.64	490	10.59	601	1091
B.W.	4.78	271	10.87	616	887
Whole worm	6.41	364	8.01	455	819

The distribution of Ca and Mg was estimated by totalling the pseudocoel and body wall contents and estimating the percentage distribution. The results are shown in table 3.15.

TABLE 3.15. The distribution of Ca and Mg in female M. dubius.

Component	Ca	Mg
P/coel	64%	49%
B.W.	36%	51%
Total	100%	100%

The Ca and Mg content of the eggs in the pseudocoel of the female was not estimated, nor was any attempt made to estimate the proportion of bound Ca and Mg, which would be far less osmotically active than the ionic elements.

3.3.6. The total contribution of the cations Na, K, Ca and Mg as chlorides, to the osmotic pressure of M. dubius was calculated and is shown in table 3.16.

TABLE 3.16. The contribution of the cations Na, K, Ca and Mg, calculated as free chlorides, to the measured osmotic pressure of female M. dubius.

Component	F.P.D.+ real osmotic pressure (mm Hg)	Osmotic Pressure contribution Total Na	Total K	Total Ca	Total Mg	Total cation (mm Hg)	Per cent contribution
P/coel	9,230	2,678	1,618	490	601	5,387	58%
B.W.	12,799	1,033	5,700	271	616	7,620	59%

F.P.D.+ freezing point depression estimate of the total osmotic pressure (see section 3.3.7)

The data shown in table 3.16 is also presented diagrammatically in figure 3.3.

These results show that at least half the osmotic pressure of female M. dubius fluids can be ascribed to the ions Na, K, Ca, Mg and Cl.

3.3.7. In order to determine the total osmotic pressure of the body wall and pseudocoel fluids of 6 week old M. dubius the freezing point depressions were estimated by the method of Jones (1941). The freezing point depressions of rat gut fluids and serum were also estimated. Six week old worms were used because the males and females are usually found close together in the anterior part of

the intestine at this age. The gut measurement provided information about the environment in which M. dubius lives, and the serum served as a check on the accuracy of the estimates obtained. (Table 3.17). The freezing point depression as osmotically equivalent solution of NaCl, of the gut fluids of 7 uninfected rats killed at 9.00 p.m. was estimated to be 274 $\pm$ 31.7 mM.

The rat host of M. dubius feeds at night in animal house cages. The result of this nocturnal activity is reflected in the freezing point depression of the rat gut fluids as those of rats killed at 3.00 p.m. have a higher mean freezing point depression (273 mM NaCl) than rats starved and killed at 9.00 p.m. (217 mM NaCl). This point must be considered when freezing point depression data is to be used to compare worms, although no consistent variation was observed. Thus the results have all been averaged and the osmotic pressure exerted by each fluid calculated. (Table 3.17).

The results are shown diagrammatically in figure 3.2 as real osmotic pressures. The differences in osmotic pressure shown in figure 3.2 are the arithmetic differences between the ideal osmotic pressure of the components. The difference is calculated from the ideal osmotic pressure (or osmotically equivalent solution, mM NaCl) rather than the real osmotic pressure because the osmotic coefficient (g) which is used to estimate the real osmotic pressure varies inversely and non-linearly with concentration (mM).

Figure 3.3 shows the contribution of the various solutes

TABLE 3.17. The freezing point depression of M. dubius components expressed as mm Hg and equivalent mM solutions of NaCl. The osmotic gradients shown are estimated in the same units.

Component	Mean F.P.D.+ (± 1 standard deviation) for rats killed at 3.00 p.m. No. estimates	Mean	Mean F.P.D.+ (± 1 standard deviation) for rats killed at 9.00 p.m. No. estimates	Mean	Mean F.P.D.+ (all estimates)	Real osmotic pressure (mm Mg)	Difference ‡ mm Hg	Difference ‡ mM NaCl
Serum rat	4	205* ± 21	4	168 ± 14	168	6,018	-2,799	(77)
Gut fluid	4	217 ± 5	4	273 ± 25	245	8,690	0	(0)
P/coel female	10	287 ± 25	12	234 ± 14	261	9,247	+597	(16)
B.W. female	12	378 ± 51	12	350 ± 14	364	12,822	+4,291	(119)
P/coel male	4	299 ± 34	12	337 ± 89	312	11,018	+2,444	(67)
B.W. male	4	563 ± 4	-	- -	563	19,590	+11,223	(318)

F.P.D.+ : Freezing point depression.

* : This serum contained lysed red cells which probably accounts for the higher freezing point depression estimate.

‡ : is the difference between the component of M. dubius and the rat gut fluid as mm Hg and the equivalent NaCl solution in(mM).

FIGURE 3.2.

Osmotic pressures of body components, rat gut and serum. The first figure is the real osmotic pressure in mm Hg and the figure in parenthesis is the mM equivalent NaCl solution. The dotted arrows represent the tendency for water movement and the osmotic gradient down which that movement would occur.

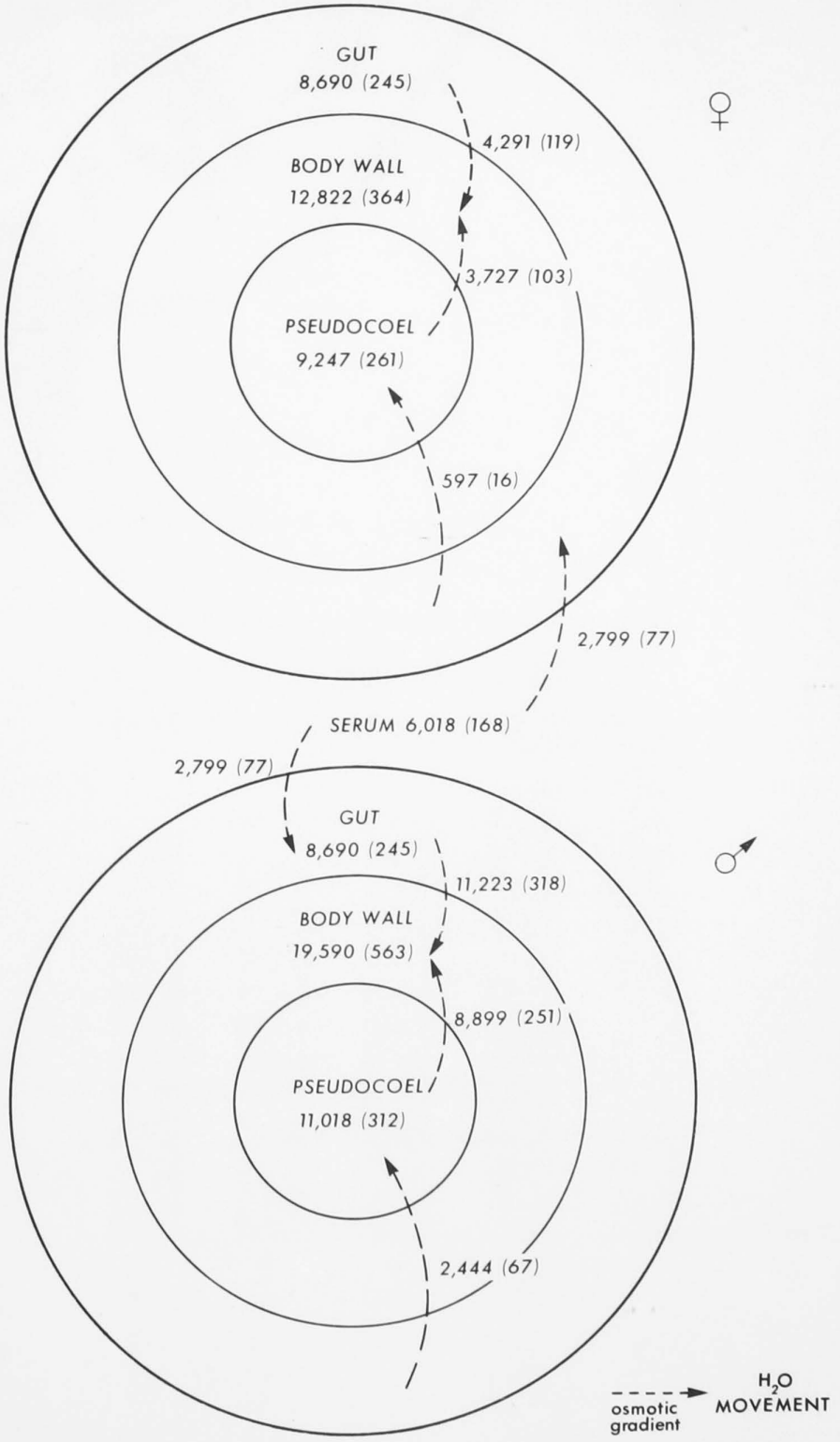
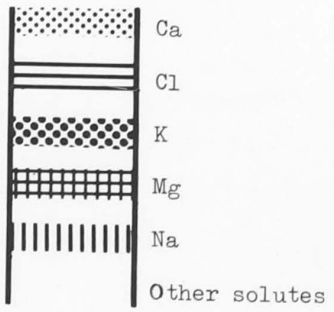

GUT
8,690 (245)
4,291 (119)
BODY WALL
12,822 (364)
3,727 (103)
PSEUDOCOEL
9,247 (261)
597 (16)
2,799 (77)
SERUM 6,018 (168)
2,799 (77)
GUT
8,690 (245)
11,223 (318)
BODY WALL
19,590 (563)
8,899 (251)
PSEUDOCOEL
11,018 (312)
2,444 (67)
osmotic gradient
H2O MOVEMENT

FIGURE 3.3.

The contribution of the ions Na, K, Mg, Ca and Cl to the real osmotic pressure of M. dubius female body wall and pseudocoel, whole male and the rat gut fluid and serum, estimated by freezing point depression. The osmotic pressure is shown as mm Hg and mOsM .

The solutes are represented as follows:



16,000
15,000
14,000
13,000
12,000
11,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
REAL OSMOTIC PRESSURE (mm Hg)
827.7
776.0
724.2
672.5
620.8
569.0
517.3
465.6
413.8
362.1
310.4
258.7
206.9
155.2
103.5
51.7
REAL OSMOTIC PRESSURE (mOsM)
HANK'S BALANCED SALT SOLUTION
RAT GUT FLUID
FEMALE PSEUDOCOEL
FEMALE BODY WALL
WHOLE MALE

to the osmotic pressures illustrated in figure 3.2.

The osmotic pressure of the cations was estimated as a percentage of the total osmotic pressure in table 3.18.

TABLE 3.18. The contribution of the total and exchangeable Na and K as chlorides, to the total osmotic pressure of the fluids of M. dubius.

Components	Total osmotic pressure (mm Hg)*	Total NaCl and KCl osmotic pressure (mm Hg)+	% of total osmotic pressure	Exchangeable NaCl and KCl osmotic pressure (mm Hg)++	% of total osmotic pressure
Serum	6,018	5,533	92**	-	-
Rat gut	8,690	5,002	58	-	-
P/coel female	9,247	3,442	37	-	-
P/coel * female	9,230	4,296	47	358	4
B.W. female	12,822	6,733	53	4,952	39
P/coel male	11,018	-	-	-	-
B.W. male	19,590	-	-	-	-

* : Freezing point depression estimate

** 92% : Figure given by Hawk (1965)

\+ : Data from Table 3.7

++ : Data from Table 3.8

3.3.8. An attempt was made to determine the distribution of Na, K and Cl in the body wall of M. dubius histochemically. The results are shown in figure 3.4, which is a highly diagrammatic view of the worm with the location of the ions shown by the density of stippling. The results may not give the true distribution of the ions because diffusion during staining cannot be prevented. However the results were consistently observed in different sections. Control sections cut from blocks of tissue soaked in distilled water did not show the same distribution of ions, although they were still present in lower concentrations.

The results show an intense band of staining for K immediately below the striped layer. K was then evenly distributed in low concentration throughout the tegument. The muscles showed quite intense even staining for K. Na was located in the greatest density at the basement membrane. Some Na was present below the striped layer and some in the muscles and supporting connective tissues.

Cl was located in the same density as the Na and K indicating that it passively accompanies these ions. The localisation of concentrations of K at the level of the striped layer suggests either that it may be a reservoir created by, or available for a cation pump, or bound in some way to the tissue components. The occurrence of concentrations of Cl in the same region suggests that the positive charge has not been neutralised by binding.

FIGURE 3.4.

Diagrammatic illustration of the distribution of Na, K and Cl in the body wall of *M. dubius*.

CM : Circular muscles.

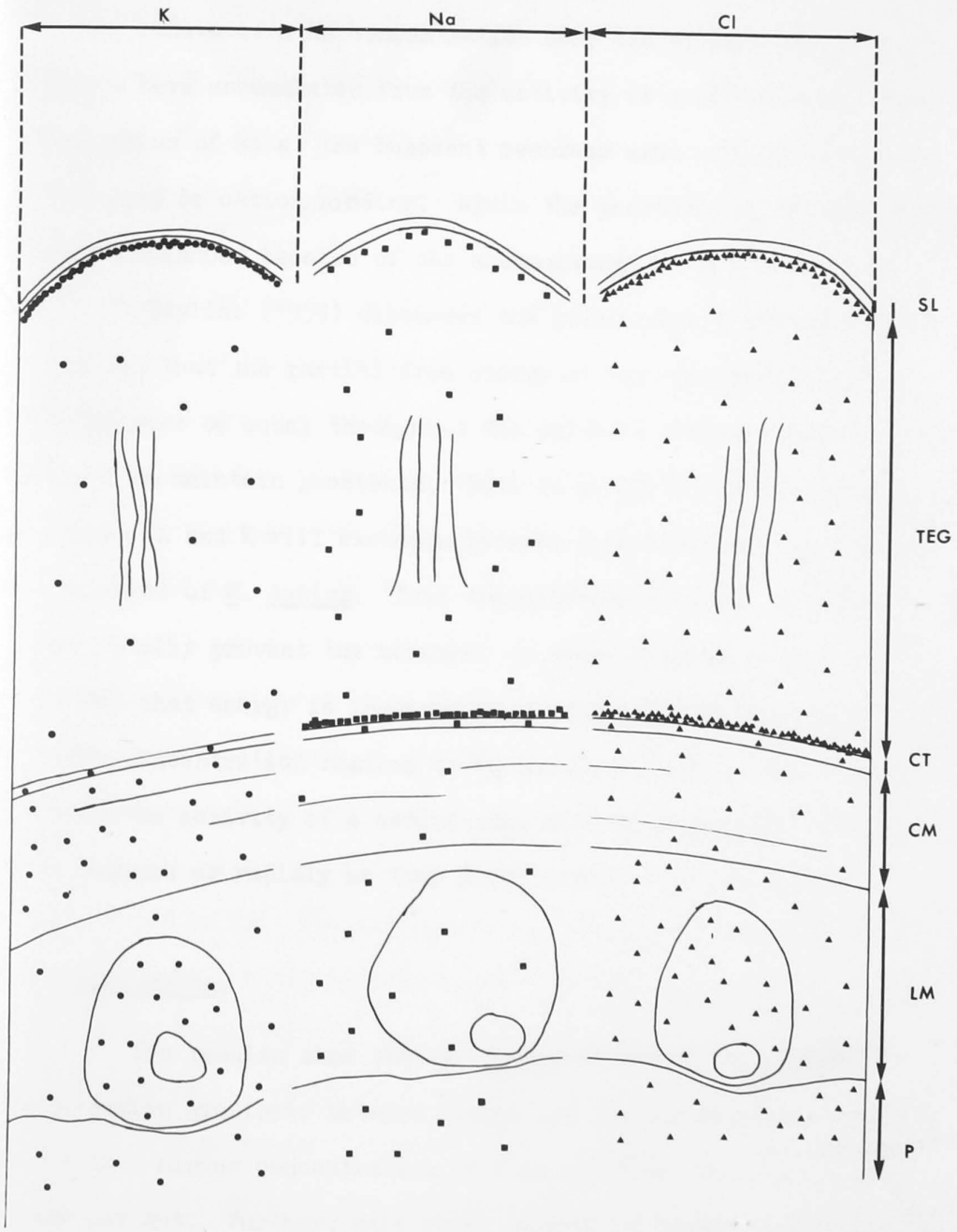
CT : Connective tissue.

LM : Longitudinal muscles.

P : Pseudocoel.

SL : Striped layer.

TEG : Tegument.


K
Na
Cl
SL
TEG
CT
CM
LM
P

There is a Na concentration near the striped layer which also may have accumulated from the activity of a cation pump. The accumulation of Na at the basement membrane again suggests either a cation pump or cation binding. Again the positive charge appears to remain unbalanced because of the accumulation of Cl ion.

Bayliss (1959) discusses the principles of diffusion and points out that the partial free energy of any component of a solution must be equal throughout the solution unless energy is expended to maintain gradients. Data is presented in chapter 4 to show that Na and K will exchange between Hanks balanced saline and the tegument of M. dubius. This demonstrates that the tegument cannot totally prevent the movement of these cations so it is concluded that energy is involved in restricting the distribution of the high concentration regions of Na and K, either by some type of bond, or the activity of a cation pump which replaces the cations in these regions as rapidly as they diffuse away from the site.

3.4. Discussion.

The results show that M. dubius maintains two major concentration gradients between itself and its environment. The worm maintains a higher concentration of K in its body wall than exists in the rat gut. Further, male worms maintain a higher concentration of K than the female worms. Both sexes maintain a lower concentration of Na than the rat gut but the males again possess the higher concentration.

Male and female M. dubius are hyperosmotic to the rat gut, and the males are hyper-osmotic to the females. Reference to table 3.7 shows that the concentration of both Na and K in whole males is higher than in whole females, (female Na 37 mM, male 64 mM, female K 106 mM, male K 225 mM). Whether or not the increased concentration of cations in the males represented the total increase in osmotic pressure cannot be accurately calculated as no Na and K analysis of the body wall and pseudocoel of males was available. The difference in osmotic pressure exerted by Na and K between whole males and females may be calculated from table 3.7. The difference in Na concentration is 27.43 mM Na, which is 1015 mm Hg real osmotic pressure, and for K 119.43 mM which is 4306 mm Hg real osmotic pressure. This gives a total difference of 5321 mm Hg. If the average osmotic pressure of pseudocoel and body wall of males and females is calculated from table 3.17, the average female osmotic pressure (including the cations in the eggs) is 11,035 mm Hg and males 15,304 mm Hg. The difference is 4269 mm Hg. The difference estimated from the cation data is 5321 mm Hg. This tentative calculation shows that the increased cation content of males could easily account for their increased osmotic pressure, and also shows that the average osmotic pressure of M. dubius cannot be obtained from the simple mean of the body wall and pseudocoel, i.e. the ratio of body wall volume to pseudocoel volume is not 1.: 1.

The reason for the higher osmotic pressure of the males is not known. It is known that the males dwell further down the

intestine than the females and it may compensate for the removal of water by the intestine and consequent increase in the osmotic pressure of their environment.

The physiological significance of the existence of the concentration gradients of Na and K is unknown, but it is significant to note that the direction of both gradients is the same as those which exist between mammalian tissue cells and their surrounding fluids. This evidence, plus that of chapter 2, shows that M. dubius is capable of active transport of the cations Na and K. The worm is also capable of retaining these ions against a concentration gradient, either by the presence of a semipermeable membrane, the activity of transport pumps, ion binding, or all three possibilities.

The results obtained from swelling female M. dubius in hypotonic sucrose shows that 58% of the pseudocoel Na is lost over two hours, while only 2% of the body wall Na is lost; this represents a loss to the whole animal of 38% of its Na. Forty three per cent of pseudocoel K is lost, and 75% of body wall K, which together represent 71% of the total K. The Na content of the body wall may have been maintained either by transfer of Na from the pseudocoel, which represents the largest reservoir of Na, or by virtue of being bound to indiffusible organic molecules within the worm. The other alternative is that Na in the body wall is retained by the electrical gradient because K, which is a smaller molecule and probably more mobile, as well as having a greater concentration gradient down which to flow, is lost. Thus the retention of some cations may be in

response to the electrical and Gibbs-Donnan equilibria which would result from loss of cations into the sucrose solution. Goodchild, Dennis and Moore (1962) give the ratio of K to Na in H. diminuta as 3.44, 2.73 and 2.23 for the anterior, middle and posterior thirds of the worm with a mean of 2.80. The mean ratio for whole female M. dubius is 2.84 and for males 3.52. Thus the balance of cations is similar for female M. dubius and H. diminuta.

The osmotic pressure of rat serum (168 mM NaCl, table 3.17) was in close agreement with the figure of 167 mM NaCl for human serum given by Hawk (1965). Robinson (1960) cites Lilienthal and Zierler (1957) who gave the concentration of the extracellular fluids of rat, cat and dog a range of values between 145 and 190 m Eq/l (145 - 190 mM NaCl). Thus the experimental figure of 168 mM NaCl is within the range of other published results, which permits greater confidence in the experimental results.

All components of M. dubius are hyperosmotic to the rat gut fluid if total cation concentration is considered and iso-osmotic if exchangeable cations are considered. This finding is in agreement with the apparent isotonicity of Hanks balanced salt solution. The pseudocoel and body wall of female M. dubius have been estimated by freezing point depression to be 597 mm Hg and 4291 mm Hg respectively, hyperosmotic to the rat gut fluids. Male pseudocoel and body wall have been estimated to be 2,444 mm Hg and 11,223 mm Hg respectively, hyperosmotic to the rat gut fluids. It has been shown that the tegument of M. dubius is permeable to water,

and Baer (1961) and King (1965) comment on the absence of an identifiable excretory system in M. dubius. Thus the method by which water movements are controlled is unknown but will be discussed in chapter 5.

The osmotic pressure of M. dubius female body wall is 53% attributable to total cation chlorides, of which 5700 mm Hg, or 5700/12799 = 44.5%, is attributable to total K-Cl. Thus K and its accompanying Cl ion alone are largely responsible for the higher osmotic pressure of the female body wall. K may be accumulated as a reservoir of ions from exchange pumping of Na and K. The creation of such a large osmotic gradient and potential water control problem would suggest that K has a further physiological role to play in M. dubius.

The exchangeable cations have been shown to be in equilibrium with the rat gut, (see table 3.8). The very high osmotic pressures determined by freezing point depression (see table 3.17) probably represent the osmotic pressure of the total cations, as the destruction of tissue to extract the fluids may result in the cations being released from their bound configuration and becoming exchangeable ions.

The Na and K concentrations of M. dubius body wall were increased by 2 days in vitro culture, in balanced salt solutions containing 137 mM NaCl and 5 mM KCl. The Na concentration of the pseudocoel decreased while the K concentration increased. This result clearly shows that K is actively accumulated or retained in

vitro because the gut contains 28 mM K and the media contain 5 mM K. Despite this decrease in external K concentration the worms have apparently increased the K concentration of both pseudocoel and body wall. Na levels have increased in the body wall and decreased in the pseudocoel. The whole worm Na content is slightly increased, indicating either a greater inflow of Na down its increased concentration gradient, (111 mM NaCl in the gut and 137 mM NaCl in culture medium) or decreased removal of Na ion.

The worms lost an average of 10% of their wet weight despite this increase in cation concentration. The loss of 10% wet weight appears contradictory to the increased concentration of alkali cations in the body wall. However if the alkali cation osmotic pressure is calculated from the whole worm analysis, the results are 4,845 mm Hg for worms from the culture and 5163 mm Hg (real osmotic pressure) for worms from the rat gut. This is a reduction of 318 mm Hg or 6%. Thus the whole worm osmotic pressure has decreased by 6% despite apparent increases in the alkali cation concentration of the body wall. This indicates that the body wall has lost water, producing a higher cation concentration, but also making the less concentrated pseudocoel of greater importance in determining the overall osmotic pressure. The result also demonstrates, however, that water content is not simply dependent on alkali cation concentration, which largely determines the higher osmotic pressure of the worm.

Ca and Mg ion are present in sufficient quantities to add

appreciably to the osmotic pressure of M. dubius. Ca and Mg as chlorides are responsible for 12% of the osmotic pressure of the female pseudocoel and 7% of the female body wall osmotic pressure. Diem (1962) suggests that normal human plasma should contain 2.4 mM Ca and 0.82 mM Mg. In whole female M. dubius the levels are 6.4 mM Ca and 8.0 mM Mg. The levels in rat plasma and the rat gut are not known.

The physiological role of Ca in M. dubius is not known. It is postulated that Ca is necessary for muscle contraction and to control membrane permeability by protein cross-linking with the divalent Ca ion. (Hawk (1965), Chambers and Chambers (1961)). Chambers and Chambers describe the pellicle of cell walls as a cement of proteinates and pectates which are insoluble as Ca salts and soluble as Na and K salts. The Ca ion thus promotes rigidity in extracellular coatings. The exact location of the Ca in M. dubius is unknown, but in the body wall Ca may be associated with the mucopolysaccharide meshwork epicuticle and the body musculature. The function of Ca in the pseudocoel is not known but may be associated with the eggs. Mg ion is an essential co-factor for many enzymatic reactions, including the oxidative pathways of glucose and almost all reactions involving ATP. Mg is also essential for the maintenance of muscular activity. Whether or not Mg has any further physiological function in M. dubius is not known.

CHAPTER 4

THE NA AND K DYNAMICS OF M. DUBIUS (ACANTHOCEPHALA).

4.1. Introduction.

Chapter 4 deals with the movements of cations through the bounding membranes of M. dubius and their distribution within the worm. The radioactive isotopes sodium-24 and potassium-42, in the forms of $Na^{24}Cl$ and $K^{42}Cl$, were used. Both isotopes which have half-lives of 15 and 12.5 hours respectively, emit gamma rays. The experiments were carried out at the Australian Atomic Energy Commission Establishment at Lucas Heights, New South Wales.

The preceeding chapters have reviewed the literature concerning cation distribution in helminths, and the author is not aware of any publications dealing with radio-active cation movements. These isotopes have been used in investigating cation movements in other animals, for example, by Levi and Ussing (1948), Harris and Burn (1949) and Keynes (1954), who demonstrated that the muscle membrane was permeable to Na ions, and provided estimates of the fluxes of Na and/or K through muscle membranes.

The earlier work reported in this thesis has suggested the existence of Na and K pumps in the tegument of M. dubius, and the maintenance of unequal distributions of both cations within the worm, and between the worm and its environment, by active transport.

Subsequent experiments were planned with three objectives in view:-

1. To determine how rapidly Na and K enter the body wall and the pseudocoel of M. dubius.
2. To determine what fraction of the Na and K present in the worm tissues are readily exchangeable for external Na and K.
3. To determine how changes in external Na and K concentrations affect the uptake and loss of Na and K.

4.2. Materials and Methods.

4.2.1. Experimental Methods. Throughout the experiments Hanks balanced salt solution was used, unless otherwise stated. This solution contains 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4.7H_2O$, 1 mM Na_2HPO_4, 1 mM KH_2PO_4, 4 mM $NaHCO_3$ and 11 mM glucose per litre. 5.5 ml cultures were normally used in 20 ml screw cap glass bottles. The incubations were performed at 37°C for 90 minutes under an atmosphere of 92% N_2 + 8% CO_2 unless otherwise indicated.

Na^{24} and K^{42} were used together in all the cultures. All cultures were labelled with 0.25 microcurie of Na^{24} and 0.25 microcurie of K^{42}. The volume of stock isotope solution added to give this content was calculated from the activity of the stock at zero time (t_0 - 1600 hours on the day of delivery of the isotopes) and the decay correction factor at the time of labelling the culture media. Decay correction factors were taken from Diem (1962). Worm content of Na^{24} and K^{42} was calculated from a formula (see appendices) and the activity corrected for isotope decay. The decay correction

was estimated from t_0 to the time of counting the sample. The counter efficiency for each isotope was determined from 0.2 ml aliquots of the culture media which were prepared volumetrically. These efficiency values were then used throughout the experiment.

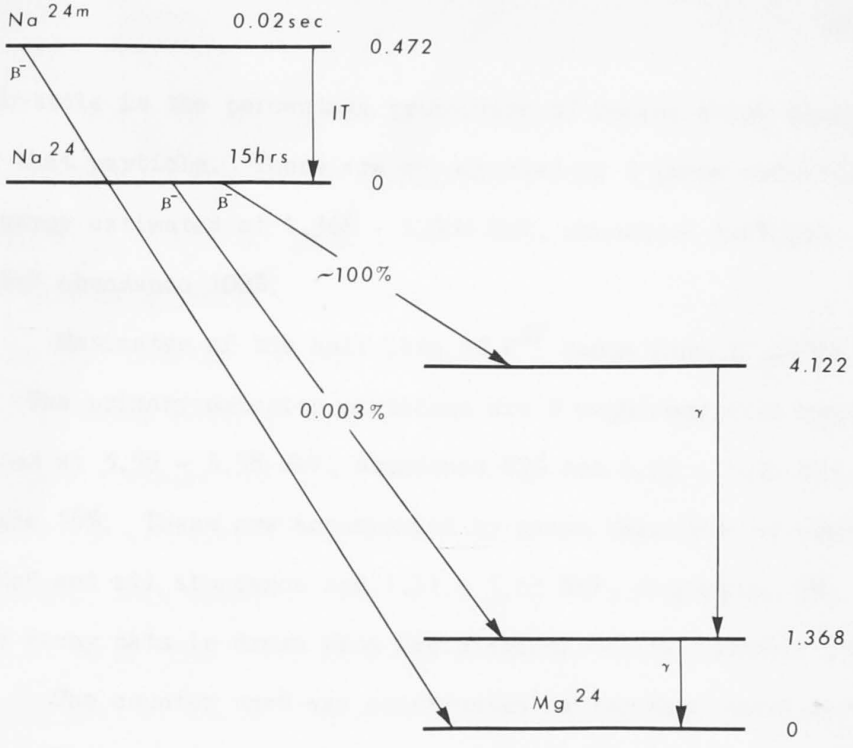
Fresh 6-8 week old worms were taken from male and female rats and weighed. The worms were then placed in the test medium for the required time, removed, rinsed in Hanks balanced salt solution, blotted dry and weighed. An end was cut off the female worms and the pseudocoel fluid squeezed out into a tared beaker and weighed. The body wall was cut longitudinally, blotted to remove pseudocoel fluid and weighed. Whole male worms were treated by the same methods. The samples were dried at 115°C for 2 hours and reweighed. They were digested with 1.0 ml of 2N - HNO_3 plus 0.25 ml of 30% $HClO_4$ and warmed on a hotplate, until a clear pale yellow solution was obtained. This process involved some fuming of the acids, so the mixture was evaporated to dryness and re-suspended in 0.5 ml of 2N - HNO_3. Na^{24} and K^{42} were assayed from 0.2 ml aliquots of the body wall, pseudocoel and whole male digests.

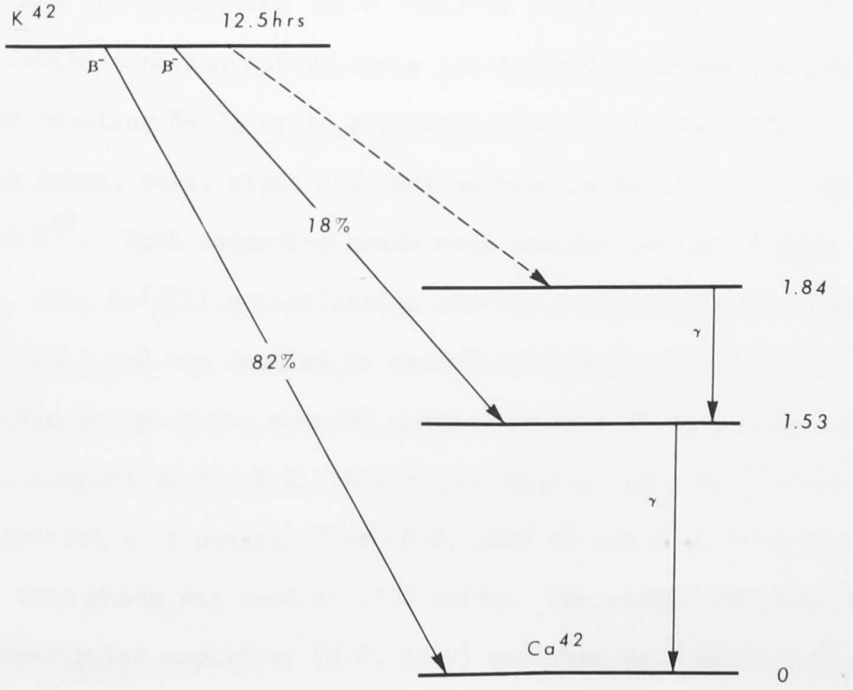
The decay characteristics of Na^{24} and K^{42} are shown in figure 4.1.

Estimates of the half life of Na^{24} range from 14.97 to 15.10 hours. The primary emission particles are 2 negatrons (beta particles). One of the beta particles possesses energy estimated at 1.390 to 1.400 million electron volts (MeV) with 100% abundance and the second pathway 4.17 MeV with abundance 0.003%. The abundance

FIGURE 4.1.

Decay schemes for Na^{24} and K^{42} (after Radiological Health Handbook (1960)).


Na 24m
0.02sec
0.472
β⁻
IT
Na 24
15hrs
0
β⁻
β⁻
~100%
4.122
0.003%
γ
1.368
γ
Mg 24
0


K 42
12.5hrs
β⁻
β⁻
18%
1.84
γ
82%
1.53
γ
Ca 42
0

of a particle is the percentage proportion of nuclei which decay by way of that particle. These are accompanied by 2 gamma radiations with energy estimated at 1.368 - 1.380 MeV, abundance 100% and 2.753 MeV abundance 100%.

Estimates of the half life of K^{42} range from 12.46 to 12.52 hours. The primary emission particles are 2 negatrons with energies estimated at 3.55 - 3.58 MeV, abundance 82% and 1.97 - 1.99 MeV, abundance 18%. These are accompanied by gamma emissions of energy 0.309 MeV and nil abundance and 1.51 - 1.53 MeV, abundance 18%. All isotope decay data is drawn from Radiological Health Handbook (1960).

The counter used was constructed at the Department of Radiation Biology of The Australian Atomic Energy Commission. The scaling and descriminating units could be connected either to a sodium iodide/thallium iodide gamma scintillation crystal, which was used for counting Na^{24}, or to a plastic phosphor suitable for counting gamma, beta, alpha and fast neutron radiation, which was used for K^{42}. Both detecting heads were mounted inside 2" lead castles. The NaI/TlI scintillation crystal surrounded a $\frac{3}{4}$" diameter $1\frac{1}{2}$" deep well and was coupled to an E.M.I.6097B photomultiplier. The plastic phosphor was mounted under a 2" wide $\frac{1}{4}$" deep well and was also coupled to the E.M.I.6097B photomultiplier. Both systems were connected to a preamplifier (N.E. 5202 A) and then to a high tension unit which was used at 1100 volts. The signal was then led to a linear pulse amplifier (N.E. 5202) and then to a differential pulse height selector (N.E. 5104B), and finally to a scaler unit.

The gain setting and pulse height analysis units were calibrated using high activity sources of Na^{24} and K^{42}. The gain was set at 4.76 and the pulse height analysis unit was set with a lower gate of 85 volts and slit width 10 volts, using the unit as a differential counter i.e. set to count the 2.76 MeV gamma emission of Na^{24}. These settings exclude most of the K^{42} beta particle energies which have averages of 0.309 and 1.53 MeV. The same gain setting was used for counting K^{42} but the counter was set for integral operation. This means that no upper gate is set on the pulse height analysis unit. The lower gate was set at 40 volts. This setting excludes most of the Na^{24} negatrons which have a maximum energy of 1.40 MeV, but includes a large proportion of the K^{42} beta particles with 3.56 Mev energy.

The instrument was thus set to count Na^{24} by its 2.76 MeV gamma emission and K^{42} by its 3.56 MeV beta particle. The pulse height analysis settings do not completely discriminate between Na^{24} and K^{42}. The Na^{24} beta particle (1.44 MeV) together with the ability of the plastic phosphor to respond to gamma radiation leads to contamination of the K^{42} count by Na^{24} and vice versa. Corrections for "cross talk" are made in the calculations. As the 3.56 MeV beta particle of K^{42} represents only 82% of the actual decay events the K^{42} counts were corrected to 100% abundance.

Na^{24} in the nitric acid extract, was counted in plastic snap-cap tubes inserted into the well of the scintillation crystal detector. K^{42} in the nitric acid extract was dried on to a 2"

aluminium planchet, and counted with the plastic phosphor. The planchets were dried on small levelling platforms under infrared lamps. Sample geometry was controlled by coating the perimeter of the planchets with perspex jelly, (perspex chips dissolved in chloroform) leaving a circle of $\frac{1}{2}$" diameter in the centre of the planchet. The sample geometry of the liquid Na^{24} count was uniform as the fluid lay in the base of the vial.

All counts were taken over 100 second periods, and were converted to counts per minute (cpm). The time of counting was recorded to the nearest 0.16 hour and the appropriate decay factor applied in the calculations.

The effect of the cardiac glycoside ouabain (Strophanthin G) on the movement of cations between M. dubius and Hanks balanced salt solution was tested. The method of Glynn (1957) was used, the glycoside being dissolved in ethanol before it was added to the medium.

Throughout the result section of this chapter the following headings and symbols are used in the tables. They are:-

Na or K concentration factor: calculated by the method shown in section 4.2.2. Difference: This is defined as the difference between the concentration factors shown in the various tables of results and the concentration factors determined in section 4.3.2 for Hanks balanced salt solution. Thus the figures presented in table 4.2 act as referents for computation in subsequent experiments. A negative difference indicates that the test value was

less than the reference value and a positive difference that the test value was greater.

P/coel F. and B.W.F. represent the pseudocoel and body wall of one female worm. Whole M. represents the result from one whole male worm.

4.2.2. Calculation methods. The method of calculation used to obtain the results shown in section 4.3 is shown in appendices 4.1 to 4.4.

In the interest of clarity the design of each experiment is given with the results. The overall experimental procedure is shown in section 4.2.1.

A recovery experiment was performed to determine the loss of Na and K due to the experimental procedure. The recovery efficiencies were:- Na, 88.89%, K, 78.83%, from which the recovery factors $\frac{100}{88.89} = 1.1250$ and $\frac{100}{78.83} = 1.2686$ were calculated. These factors have been applied to all the results shown in section 4.3. The tables show the concentration factors

$$\frac{\mu M\ Na^{24} \text{ or } K^{42}/g \text{ worm water}}{\mu M\ Na^{24} \text{ or } K^{42}/g \text{ culture medium}}$$

for the worms under various experimental conditions and compare the results with the reference values established in section 4.3.2. They are presented in this way because the concentration of Na^{24} and K^{42} in the media varied considerably and presentation as ratios allows direct comparison of the figures.

All the results in this chapter were obtained over one

month at the Australian Atomic Energy Commission Establishment at Lucas Heights, N.S.W. Only limited numbers of M. dubius were available so the experiments have not been replicated and frequently reliance is placed on the result obtained from only one worm.

The proportion of the Na and K in the tissues which was readily exchangeable in Hanks balanced salt solution was estimated in the following way. The calculation is given for K but applies to Na in the same way. Let the concentration of K^{39} and K^{42} in the medium equal K^{39}_c μM/ml and K^{42}_c μM/ml respectively. Let the concentration of K^{39} and K^{42} in the worm after the incubation be K^{39}_w and K^{42}_w. Le the concentration factor between the worm and the medium at the end of the incubation be A, where A

$$= \frac{Na^{24}_w \text{ or } K^{42}_w \ (\mu M/ml)}{Na^{24}_c \text{ or } K^{42}_c \ (\mu M/ml)}$$

Then the total K concentration of the medium is $K^{39}_c + K^{42}_c$. Thus the amount of K absorbed by the worm from the culture is $(K^{39}_c + K^{42}_c) \times A$ μM/ml. Thus assuming that the worm has not lost any K and that the medium concentration has not been significantly altered by the removal of K by the worm, the total K concentration of the worm is the static concentration given in chapter 3 plus $(K^{39}_c + K^{42}_c) \times A$.

The static levels, (K^{39}_w) are:-

	Pseudocoel (μM/ml)	Body wall (μM/ml)	Whole male (μM/ml)
Na	74	28	64
K	44	159	225

Thus, for example the new K concentration of the body wall would be $K^{39}_{w} + (K^{39}_{c} + K^{42}_{c}) \times A$, or $159 + (K^{39}_{c} + K^{42}_{c}) \times A$ $\mu M/ml$.

The "specific activity" of K^{42} in the body wall is:

$$\frac{K^{42}_{w}}{159 + (K^{39}_{c} + K^{42}_{c}) \times A} = S.A._{bw}.$$

The specific activity of K^{42} in the medium is:

$$\frac{K^{42}_{c}}{K^{39}_{c} + K^{42}_{c}} = S.A._{c}.$$

The proportion of body wall K which is exchanged is thus given by:

$$K_{\text{exchangeable}} = \frac{S.A._{bw}}{S.A._{c}}$$

or $$K_{\text{exchangeable}} = \frac{K^{42}_{w}}{159 + (K^{39}_{c} + K^{42}_{c})} \times \frac{K^{39}_{c} + K^{42}_{c}}{K^{42}_{c}}$$

4.3. Results.

4.3.1. An experiment was performed to determine the fractions of Na and K which were exchangeable in a given time. The results are shown in table 4.1 and figures 4.2 and 4.3.

TABLE 4.1. The mean proportion of the Na and K in M. dubius whole males, female body wall and female pseudocoel which was exchanged after incubation for various time intervals in Hanks balanced salt solution. (The means were calculated from data for 3 worms and are shown ± 1 standard deviation).

Time (mins)	B.W. F.		P/coel F.		Whole M.	
	Na	K	Na	K	Na	K
5	16.1±14.1	16.4± 6.2	5.5± 9.6	0±0	15.8± 3.5	13.0± 7.2
15	34.4± 7.5	28.9±22.8	5.2± 8.9	4.6± 7.9	30.1±38.8	4.0± 6.9
30	25.6±13.8	40.5±22.7	6.5± 6.4	13.4±23.7	29.1±36.6	35.9±11.5
60	26.9± 7.2	48.7±21.7	8.1± 8.3	9.5±16.9	17.3± 6.4	37.7±15.1
120	32.6± 2.5	81.2± 0.7	10.0±11.2	13.4±23.2	15.1± 8.5	56.8±16.4
240	21.9± 7.9	72.4±15.7	16.5± 4.3	61.4±14.5	16.6± 7.1	69.5± 9.6
Mean	26.2± 6.8	-	8.6± 4.3	-	20.7± 6.9	-

Except for pseudocoel Na and K the results shown in table 4.1 approached a steady state in 4 hours. The Na exchange was more stable after 4 hours in female body wall and whole males than K exchange. Female pseudocoel exchanged 8% ± 4% of its Na in 4 hours while the body wall exchanged 26% ± 7%. In the whole male 21% ± 7% of Na was exchanged in 4 hours. Female pseudocoel exchanged 13% ± 23% of its K in 2 hours and 61% ± 15% in 4 hours. This may indicate that there are 2 pools of K in the pseudocoel which exchange at different rates. The two pools may be the pseudocoel fluid and the

FIGURE 4.2.

Changes in the percentage proportion of total K exchanged by M. dubius female body wall and pseudocoel and entire male, with time.

(All lines drawn by inspection. The vertical lines represent $\pm$ 1 standard deviation).

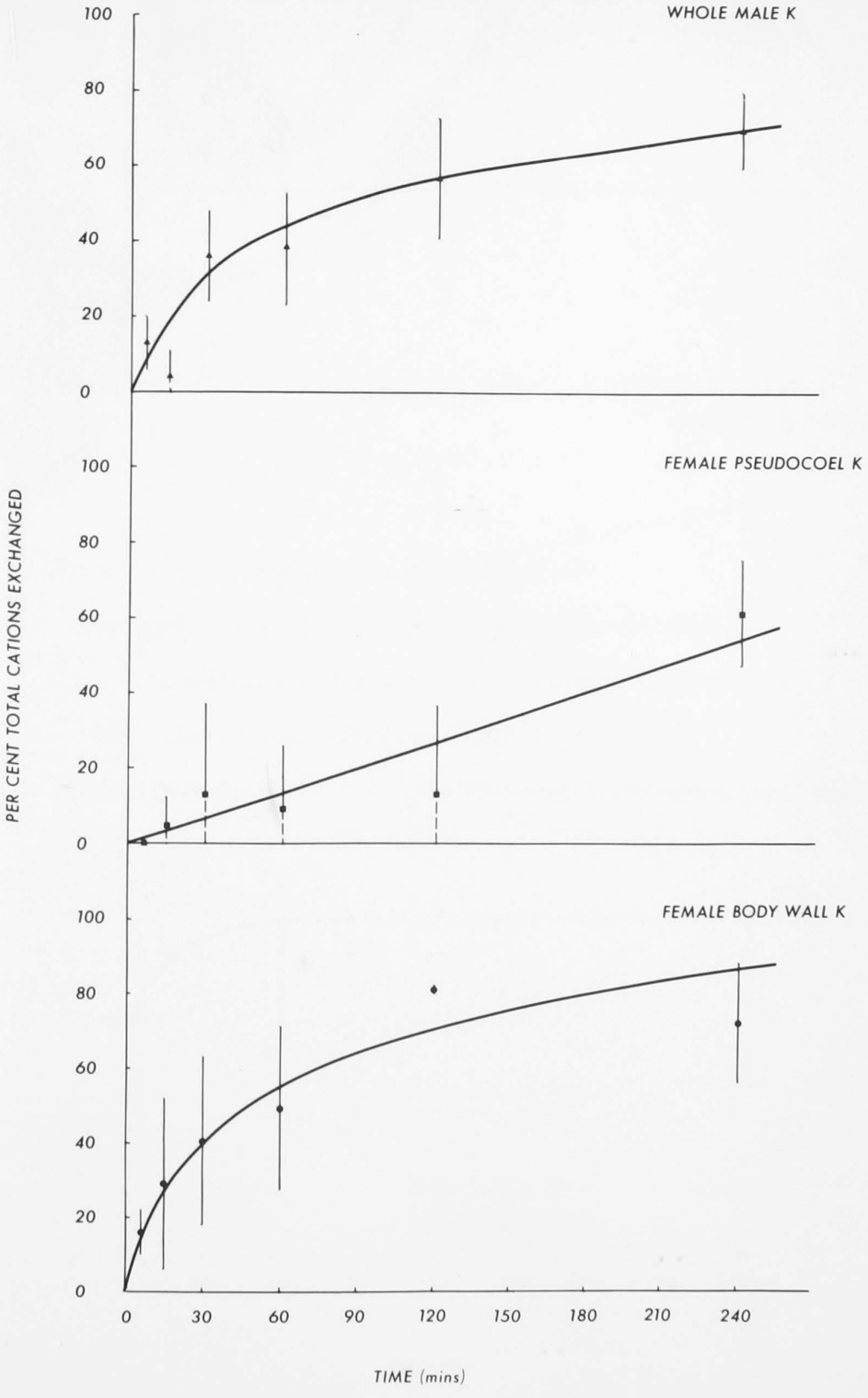
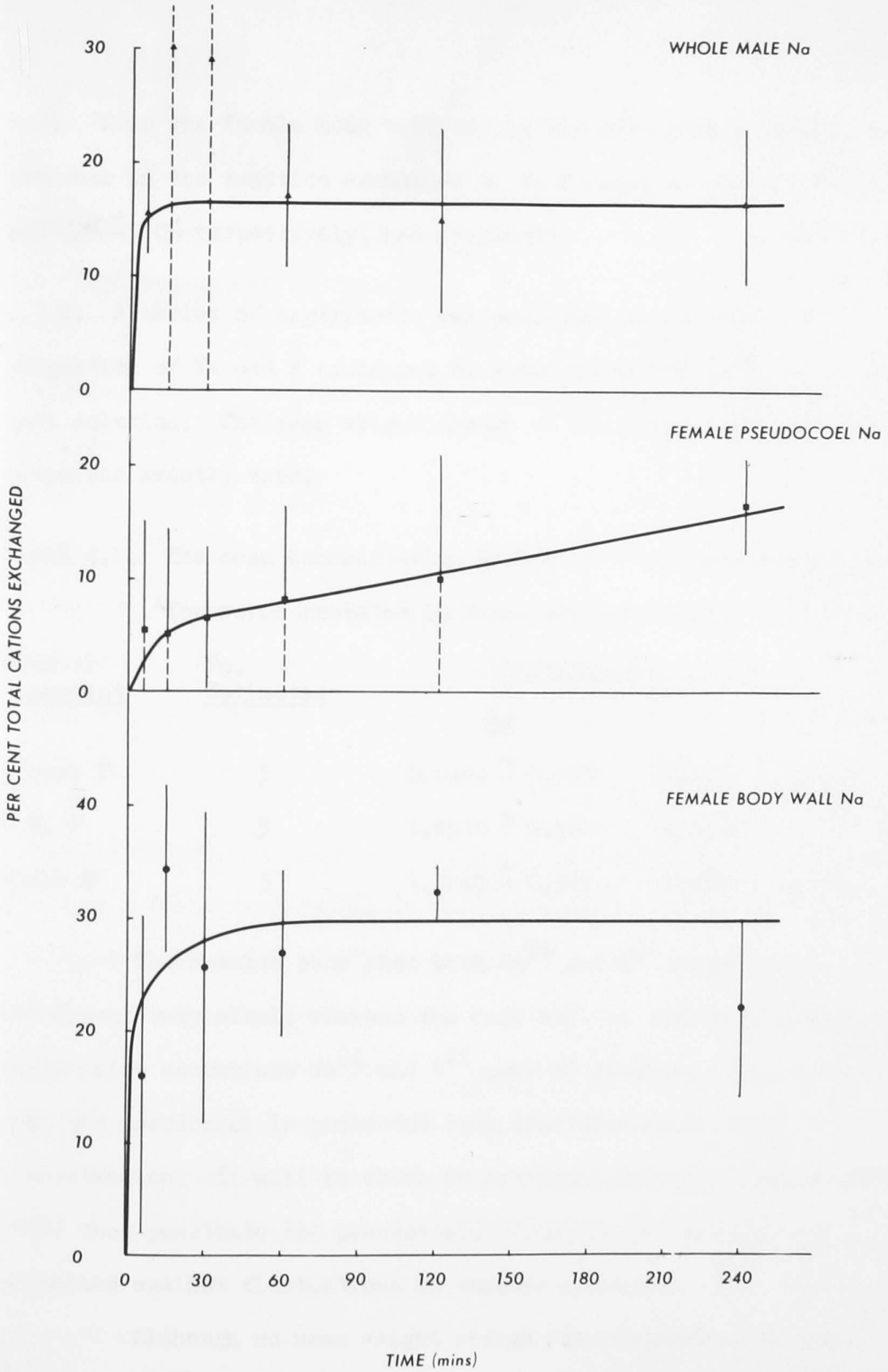

WHOLE MALE K
FEMALE PSEUDOCOEL K
FEMALE BODY WALL K
PER CENT TOTAL CATIONS EXCHANGED
100
80
60
40
20
0
0
30
60
90
120
150
180
210
240
TIME (mins)

FIGURE 4.3.

Changes in the percentage proportion of total Na exchanged by M. dubius female body wall and pseudocoel and entire male with time.

(All lines drawn by inspection. The vertical lines represent $\pm$ 1 standard deviation).


WHOLE MALE Na
FEMALE PSEUDOCOEL Na
FEMALE BODY WALL Na
PER CENT TOTAL CATIONS EXCHANGED
TIME (mins)
0
10
20
30
40
0
30
60
90
120
150
180
210
240

eggs. K in the female body wall and in the male show a steady increase in the fraction exchanged up to 4 hours at which 72% ± 16% and 70% ± 10% respectively, had exchanged.

4.3.2. A series of experiments was performed to determine the proportion of Na and K exchanged by worms incubated in Hanks balanced salt solution. The mean weight change of the worms in the culture media was exactly zero.

TABLE 4.2. The mean concentration factor (± 1 standard deviation) for worms incubated in Hanks balanced saline for 90 mins.

Worm or Component	No. Estimates	Concentration Factor	
		Na	K
P/coel F	5	0.0464 ± 0.103	0.3602 ± 0.259
B.W. F	5	1.4576 ± 0.161	2.4380 ± 0.354
Whole M	3	1.1243 ± 0.569	1.5555 ± 0.152

The results show that both Na^{24} and K^{42} enter the pseudocoel very slowly whereas the body wall of female M. dubius and whole males accumulate Na^{24} and K^{42} over 90 minutes. This indicates that the pseudocoel is protected from fluctuations in salt concentration. It will be shown in section 5.3.3 that trituim oxide (H_2^3O) does penetrate the pseudocoel. Thus the pseudocoel is not protected against fluctuations in osmotic pressure.

Although no mean weight change has occurred, individual weight changes of +0.8% to -0.8% did occur. If cations were being

accumulated to give a higher cation concentration the osmotic pressure of the worms would have increased. It was shown in chapter 2 that the worms are permeable to water, so a weight increase would have followed cation accumulation. This has not occurred so the cations entering the worm must be exchanged for some other positively charged particle, or bound and rendered osmotically inactive within the worm.

Thus Na enters the worm to reach a greater concentration than in the medium which indicates active accumulation of Na. K behaves similarly: all the accumulation of K is against its concentration gradient as the initial concentration of K in the female body wall is 159 mM K and in Hanks balanced salt solution 5 mM K. The gradient in the whole male is from 5 mM K to 225 mM K.

4.3.3. The cardiac glycoside ouabain, an inhibitor of Na transport in other animals, was tested to determine whether it affected accumulation of cations by M. dubius. A control group incubated in Hanks balanced salt solution containing the same volume of ethanol as the test group was included.

The results showed that only the female body wall of one worm underwent a significant reduction in the accumulation of both Na and K. The concentration of ouabain was 2.18×10^{-4} mg/ml. The pseudocoel of this worm and a male worm incubated in the same medium were not affected. The failure of all the lower concentrations of ouabain to inhibit the cation exchange suggests either that the uptake

of cations is insensitive to ouabain or that the drug has been unable to reach its site of action. The significance of the single detectable difference is unknown.

4.3.4. Experiments were designed to determine the affect of differences in tonicity of the culture medium on Na and K uptake. Hanks balanced salt solution was made hypertonic by the addition of 322 mg NaCl per 100 ml. This increased the concentration of Na from 137 mM to 192 mM, and raised the real osmotic pressure from 5739 mm Hg to 7838 mm Hg, which caused an average weight loss of 12.5% by the worms.

The results showed significant differences, only for the body wall of one female worm for both Na and K. Otherwise reduced uptake of both cations occurred in all cases but the differences were not significant ($p = 0.050$). This result can be interpreted as a consequence of the shrinkage of 12.5%, with the concomitant increase in the concentration of the endogenous cations and corresponding rise in the internal osmotic pressure.

Worms were incubated in Hanks balanced salt solution diluted by the addition of 26 mls of distilled water to 100 mls of Hanks saline. This reduced the real osmotic pressure of the solution from 5739 mm Hg to 4555 mm Hg and resulted in an average weight increase of 17.2%. The results are shown in table 4.3.

TABLE 4.3. The effect of a decrease in the osmotic pressure of the culture medium on the accumulation of Na and K by M. dubius at 37°C.

Time (mins)	Worm or Component	Na conc. factor	Diff.	Sig.	K conc. factor	Diff.	Sig.
15	P/coel F	0.050	+0.0036	N.S.	0.006	-0.3542	N.S.
	B.W. F	0.452	-1.0056	***	0.552	-1.8860	**
30	P/coel F	0	-0.0464	N.S.	0.230	-0.1302	N.S.
	B.W. F	0.729	-0.7286	*	0.878	-1.5600	*
45	P/coel F	0.152	+0.1056	N.S.	0.055	-0.3052	N.S.
	B.W. F	0.875	-0.5826	*	1.425	-1.0130	N.S.
60	P/coel F	0	-0.0464	N.S.	0.157	-0.2032	N.S.
	B.W. F	0.694	-0.7636	*	1.553	-0.8850	N.S.

The results show that after 45 and 60 minutes the accumulation of K was reduced but the reduction was not significant ($p = 0.050$). The accumulation of Na was significantly reduced in the body wall, but not in the pseudocoel. The results from preliminary experiments, which are not included in section 4.3, showed that the accumulation of cations from Hanks balanced saline after 30 and 60 minutes was not significantly different from the accumulation at 90 minutes. Incubation in hypo-osmotic Hanks balanced saline caused an average swelling of 17.2% and a significant decrease in Na accumulation by the body wall of female worms. However K accumulation was not significantly reduced in either the body wall

or pseudocoel.

4.3.5. This section reports the results of incubating labelled M. dubius in Hanks balanced salt solution (osmotic pressure 5739 mm Hg at 37°C) hypotonic Hanks (Hanks + 26 ml distilled water/100 ml Hanks producing an osmotic pressure of 4,555 mm Hg at 37°C and a Na concentration of 100 mM) and hypertonic Hanks (Hanks balanced salt solution + 322 mg NaCl/100 ml producing an osmotic pressure of 7838 mm Hg at 37°C and a Na concentration of 192 mM). The worms were incubated in labelled Hanks balanced salt solution for 90 minutes and then transferred to the modified media for 90 minutes. Cation contents of the worms after the second incubation are shown in table 4.4.

TABLE 4.4. The cation content of M. dubius after 90 minutes in Hanks, hypertonic Hanks and hypotonic Hanks.

Culture medium	Worm or component		Na conc. factor	Diff.	Sig.	K conc. factor	Diff.	Sig.
Standard Hanks	Whole	M	0.744	-0.3803	N.S.	0.912	-0.6435	N.S.
	Whole	M	0.444	-0.6803	N.S.	0.627	-0.9285	N.S.
Hypertonic Hanks	P/coel	F	0.161	+0.1146	N.S.	0.156	-0.2042	N.S.
	B.W.	F	0.711	-0.7466	*	1.314	-1.1240	*
	Whole	M	0.493	-0.6316	N.S.	0.996	-0.5595	N.S.
Hypotonic Hanks	P/coel	F	0.024	-0.0224	N.S.	0.137	-0.2232	N.S.
	B.W.	F	0.538	-0.9196	**	0.906	-1.5320	*
	Whole	M	0.524	-0.6003	N.S.	0.762	-0.7935	N.S.

The results show that reincubation in Hanks balanced salt solution has not significantly affected the Na^{24} and K^{42} of male worms. Incubation in hypotonic medium has caused a greater loss of Na^{24} and K^{42} than in hypertonic medium. Male worms are unaffected by all those media. The mean weight changes (%) were -2.8% for Hanks, -15.2% for hypertonic Hanks and +10.9% for hypotonic Hanks.

The percentage loss of Na^{24} and K^{42} from the worms is shown in table 4.5.

TABLE 4.5. The percentage loss of labelled cations from M. dubius incubated in Hanks balanced salt solution, hypertonic and hypotonic media.

Culture Medium	No.	Na	K
Standard Hanks	Male 1	47.5%	36.8%
	Male 2	40.2%	32.4%
Hypertonic Hanks	Female 3	25.2%	17.6%
	Male 4	68.6%	26.9%
Hypotonic Hanks	Female 5	38.1%	15.8%
	Male 6	33.3%	25.7%

(The method of calculation is shown in appendix 4.4)

These results all show a detectable loss of Na^{24} into the medium. The hypertonic medium has caused a greater loss of Na^{24} from the males than either Hanks balanced salt solution or the hypotonic medium. Unfortunately no females were available for the control group. The hypotonic medium has caused a greater loss of

Na^{24} from females than from hypertonic medium. K^{42} has been lost equally from hypotonic and hypertonic media in both cases there has been a smaller loss than in Hanks balanced salt solution.

The concentration gradients which would exist between whole worms and these solutions are as follows:-

	Na (mM)			K (mM)		
	Worm		Medium	Worm		Medium
	Hanks			Hanks		
Female	37	←	137	106	→	5
Male	64	←	137	225	→	5
	Hypertonic Hanks			Hypertonic Hanks		
Female	37	←	192	106	→	5
Male	64	←	192	225	→	5
	Hypotonic Hanks			Hypotonic Hanks		
Female	37	←	100	106	→	3.6
Male	64	←	100	225	→	3.6

The gradient is indicated by the direction of the arrows.

The rank order of the solutions for Na gradient into the worm in decreasing order is hypertonic Hanks, Hanks, hypotonic Hanks and the order of outward K gradients is hypotonic Hanks and Hanks which has the same gradient as hypertonic Hanks. The greatest Na loss was in the hypotonic medium which had the smallest gradient. The greatest K loss is in Hanks which had a smaller gradient than the hypotonic medium. Thus Na and K movements are not a simple function of the concentration gradient. This suggests that the worm have at least some control over their cation exchanges.

4.4. Discussion.

Na^{24} and K^{42} have frequently been used to determine the nature of the cation balances between animal tissues and extracellular fluids, but as far as the author is aware no such studies have previously been attempted with helminths. The available data on the static distribution of cations in helminths has been reviewed in chapter 3. The experiments presented in chapter 4 were designed to define the cation movements in M. dubius under a number of experimental conditions. The amount of data available for any one set of experimental conditions is very limited, and for this reason the statistical analysis has been confined to "t" tests between the final results.

The efficiency of counting for both isotopes was low because of their decay characteristics. Na^{24} decays via negatrons of energy 1.4 MeV and 4.17 MeV. K^{42} also decays via 2 negatrons of energy 1.9 MeV and 3.6 MeV. It would be almost impossible to differentiate between the counts of Na^{24} and K^{42} using their 1.4 and 1.9 MeV negatrons because of the small energy difference. For this reason K^{42} was counted by its 3.6 MeV beta particle. The 4.17 MeV beta particle of Na^{24} has abundance of only 0.003% so it did not interfere with the count obtained. The plastic phosphor used had a density of 1.032, and is barely capable of stopping and producing scintillations from a negatron of energy 3.6 MeV. Only a low counting efficiency was therefore possible.

Inability to distinguish between the 1.37 MeV gamma

emission of Na^{24} and the 1.52 MeV gamma emission of K^{42} made it necessary to count Na^{24} by its 2.75 MeV gamma emission. The depth of NaI/TlI crystal surrounding the sample is readily penetrated by a 2.75 MeV gamma emission resulting in low counting efficiency.

The low counting efficiency for the isotopes, coupled with the movement of only small amounts of isotope into the worm, and the relatively low specific activities of the isotopes used, causes the number of counts recorded /100 seconds to be quite low. Thus the potential counting error of most of the samples was between 10 and 20%.

The proportion of Na and K exchanged by the various compartments may have been affected by any of the factors discussed in chapter 3, i.e. the time of day at which the rat is killed, the sex of the rat, the number and sex of the worms in the gut, and the age of the worms. The variability of the individual responses is shown in table 4.2. Thus all of these factors, and their interactions, could combine to vary the response of the worms to incubation in Hanks balanced salt solution. The overall results are that 26% of body wall Na, and 72% of body wall K are exchanged in 4 hours. 8% of pseudocoel Na is exchanged and 61% of K is exchanged in 4 hours. The Na and K content of the whole male was 21% and 70% exchanged respectively at 4 hours. These results indicate that a part of the Na and K in M. dubius exchanges relatively slowly or not at all under the conditions of the experiment.

Table 3.10 shows that some cations are retained by female

M. dubius in hypotonic sucrose. 58% of pseudocoel Na and 43% of pseudocoel K were lost. The above result shows that 8% of pseudocoel Na and 61% of pseudocoel K readily exchange. Thus the female worm has lost some of its apparently non-exchangeable Na in sucrose but has retained exchangeable K in sucrose. The body wall loss in sucrose was 2% Na (c.f. 26% above) and K loss was 75% (c.f. 72% above). These results show a wide spread in the proportion of cation, retained but all the results show that some of the cations of female M. dubius are bound, either electrostatically or to proteins or other non-diffusable molecules.

Female M. dubius lost 38% of their total Na in sucrose (c.f. 21% of male Na exchanged in Hanks balanced saline) and 71% of their total K (c.f. 70% of male K exchanged in Hanks balanced saline). Thus male and female worms exchange very similar proportions of their total K.

The concentration factors given in table 4.2 show that Na and K concentration of M. dubius has increased due to inflow of these ions from the medium. As there has been no change in weight i.e. the osmotic balance remains unaltered, the incoming Na and K must be bound immediately, perhaps in the same way as the non-exchangeable cations already present. This situation is demonstrated by table 3.11, which shows the Na and K concentration in M. dubius after 2 days in vitro culture in media containing 137 mM Na and 5 mM K. Pseudocoel K and body wall Na and K have all increased. Pseudocoel Na alone has decreased. Thus the apparent cation balance in Hanks balanced

salt solution after 90 minutes is still apparent after 2 days *in vitro* culture.

The accumulation of Na and K by whole males and the body wall of females takes place against a concentration gradient, but since the electrochemical gradient is not known, the existence of an active transport mechanism cannot be proven. The results given in chapter 3 show that there is a lower concentration of Na in the pseudocoel and body wall of females, and in entire males, than in the rat gut, which suggests the existence of an outward transport of Na. However it has now been shown (see also chapter 2) that over 90 minutes the worms accumulate Na. Thus either the worms have adjusted to a new cation equilibrium in the Hanks balanced salt solution or the outward flux of Na continued beyond 90 minutes to recover the cation balance which existed in the rat gut. There was no evidence at 4 hours that the internal concentration of Na was decreasing. Thus it is suggested that *M. dubius* reaches a new cation equilibrium in Hanks balanced salt solution.

The cardiac glycoside ouabain, a widely used inhibitor of Na transport, had no consistent statistically significant effect on the exchange of Na by *M. dubius*. This may be because it did not penetrate the surface membrane to reach the cation pump. The author has been unable to inhibition transport and movement by *M. dubius* using the cardiac glycosides Scillarin A and Strophanthin G, and cyanide and 2, 4-dinitrophenol. This may reflect the efficiency of the bounding membranes to exclude, from the worm, any substance for

which it is not equipped with a transport mechanism. CN inhibition was tested in chapter 2. The failure of CN to inhibit cation transport may have been due to the predominantly anaerobic electron transport system of this worm, (Bryant and Nicholas (1965)), or to the failure of CN to penetrate the worm.

Hypertonic media had no consistent effect on the movement of cations although the shrinkage of the worms may have masked the effect. The shrinkage would have resulted in an increased concentration of endogenous cations which could in turn have resulted in a smaller proportionate exchange of cations. Thus per cent exchange of total cations may have been less than the concentration factor indicated.

Hypotonic media, which resulted in 17% swelling, significantly reduced the intake of Na but did not affect the intake of K at 45 and 60 minutes. K is present in M. dubius in greater concentrations than Na, which may indicate that internal K is of greater importance to the integrity of the worm. It is not unexpected, therefore, that the intake of Na should be reduced to a greater extent than the intake of K if the worm is attempting to reduce its internal osmotic pressure to prevent further swelling. This conclusion is further supported by the comments of Tosteson (1963) who states that most cells maintain their internal hydrostatic pressure by the actively maintained distribution of Na and K ion. Thus it is possible that M. dubius, which inhabits a region which may suffer considerable osmotic variation, and is equipped with

cation pumps, may possess a mechanism for eliminating Na, or rather causing the balances of Na fluxes to be outward.

The loss of Na^{24} and K^{42} from labelled worms reincubated in Hanks, hypotonic Hanks and hypertonic Hanks was demonstrated. The result from hypotonic and hypertonic media is difficult to interpret, but shows that the cation movements are not simply a function of concentration gradients.

Only a limited number of general conclusions may be drawn from the above results because the small number of worms used results in difficulties in interpretation. There is a constant turnover of the alkali cations, Na and K, between M. dubius and its environment. Secondly the rates of turnover can be significantly modified by varying the incubation conditions, and thirdly concentration gradients do not wholly determine the cation movements.

The results show that the pseudocoel is relatively stable with regard to fluctuations in cation content. The pseudocoel is penetrated by tritiated water (see chapter 5) but would provide an environment with a stable cation balance for the development of acanthors.

CHAPTER 5

THE WATER BALANCE OF M. DUBIUS (ACANTHOCEPHALA)

5.1. Introduction.

Chapter 5 of this work will consider the movements of water into, and out of M. dubius, and the distribution of absorbed water within the animal.

The controlled movement of water is essential for the correct functioning of cells and tissues. Tostesen (1963) discussed the osmotic consequences of failure of the outward Na pump in tissue cells, which leads to lysis of the cell in media with a high Na concentration. This serves to highlight the importance of cation distribution in the control of water movements. Tostesen points out that if an osmotic gradient is established across a cell membrane it is neutralised by the extremely rapid movement of water through the membrane. The living bounding layer of M. dubius is a membrane covered only by the fibrous epicuticle so the movement of water between the worm and its environment may be expected to be rapid.

Some organ structures, notably the vertebrate kidney and bladder, depend on the impermeability of cell membranes for their successful operation. Hays and Leaf (1962) studied the movement of water through the isoated toad bladder. They demonstrated that the toad bladder is relatively impermeable to water in the absence of vasopressin, and that this hormone induces a change in the bladder

wall and allows a flow of water, which is proportional to the osmotic gradient, across the wall. Thus the ability of the bladder to store its hypertonic urine in an undiluted state depends on the control of passive water movements by the cells of the bladder wall.

Little and Robinson (1967) suggested some consequences of sub-lytic cell swelling. They suggested that swelling may result in movement and disorientation of cytoplasmic structures and the topography of multienzyme systems, which would produce disruption of metabolic processes. Swelling would also result in dilution of pools of metabolic intermediates which would reduce reaction rates. These reasons would explain the necessity for closely controlled cell volumes.

Robinson (1954) postulated the existence of water pumps which were independant of the activity of cation pumps and the distribution of solutes in the tissues. This hypothesis is not widely accepted. Csáky (1965) states that present evidence favours the passive movement of water down its own concentration gradient, there being no active transport of water up an activity gradient. Robinson's hypothesis of active water transport is invalidated by Tosteson's (1963) evidence that any difference in osmotic pressure in naked cells is very rapidly removed by diffusion of water. The rapid diffusion of water may not occur in tissues with mechanical support, e.g. the hydrostatic skeleton of Ascaris sp. (Harris and Crofton (1957)).

The directional movement of water may be achieved by

mechanisms similar to that proposed by Davenport (1966). He suggested that water may be induced to flow into the absorptive cells of the intestine down a concentration gradient established by the Na pump. The luminal surface of these cells is relatively impermeable to Na ions, while the serosal side is relatively permeable. Thus Na ion transported through the luminal surface may rapidly diffuse into the interstitial fluid down the concentration gradient. Water flows from the intestinal lumen into the absorptive cell along the concentration gradient. The inflow of water creates a hydrostatic pressure within the cell, which is relieved by the drainage of water into the interstitial fluid, as there is no chemical gradient exerted between the cell and these fluids. Thus water movement may be induced by the creation of osmotic and hydrostatic pressure gradients. Similar "two - membrane theories" have been postulated for the water resorptive capabilities of the perineurium of Carausius morosus and Periplaneta americana, and the rectal papillae of Calliphora sp., by Maddrell and Treherne (1967) and Berridge and Gupta (1967) respectively. Maddrell and Treherne (1967) examined the perineurium of two insect species and detected the presence of minute canals in the perineurium which were closed at the inner margin and open at the outer end. These authors suggested that water would be transported out of the nerve by creation of a high concentration of Na ion in the closed end of the canal causing an inflow of water, which would then drain away from the nerve under the influence of the hydrostatic pressure

gradient. A similar system was proposed by Berridge and Gupta (1967) for the production of hypertonic urine by the rectal papillae of the blowfly. These authors further suggested the presence of an antidiuretic hormone to control the final tonicity of the urine by controlling the permeability of the plasma membrane to water. It is postulated that the permeability of membranes is controlled by varying the size and abundance of the membrane pores.

All the above references deal with the physiological aspects of water movement in tissues. Papers by Dick (1959), Løvtrup (1963) and Lea (1963) give highly mathematical accounts of diffusion through the protoplasm of cells, through the surface of cells, and the movement of water through long narrow pores respectively.

Thus the movements of water through membranes and tissues are affected by osmotic gradients, membrane permeability and hormone interactions, temperature and activation energy. It has been shown in the previous chapters that M. dubius is hyperosmotic to its environment with respect to total osmotic pressure (see chapter 3) and must therefore possess some means of controlling water movements. It was therefore necessary to trace the movements of water between M. dubius and its environment under physiological conditions, i.e. under conditions simulating those in the rat gut especially with reference to osmotic gradients and temperature. It was known that the bounding membrane of M. dubius is permeable to water under the experimental conditions used in chapter 2. The osmotic gradient was known and there were no known hormone

interactions. Thus the following experiments were designed to estimate the rate at which water enters and leaves M. dubius and the distribution of assimilated water within the worm.

Tritium oxide, or tritiated water, H_2^3O, behaves in essentially the same way as water, H_2^1O, and can be used to trace its movements. Its ability to ionise by proton transfer to form H_3O^+ and OH^- ions can lead to proton enrichment of biological molecules with tritium. Evidence is presented in section 5.3 that the tritium does not exchange to any significant degree for hydrogen in M. dubius.

Results are presented in section 5.3 which attempt to determine 6 parameters of water movement within M. dubius and between the worm and its environment. The parameters are:-

1) The rate of penetration of water into M. dubius from Hanks balanced salt solution, and the rate of water loss from M. dubius into Hanks balanced salt solution i.e. the rate of exchange of water.

2) The proportion of water in M. dubius which is exchangeable with water in Hanks balanced salt solution and how long it takes for this exchange to occur.

3) Changes in water relationships during amino acid assimilation.

4) Site of water uptake and secretion.

5) The activation energy of water uptake and loss, to attempt to characterise the mechanism involved.

6) The permeability constant of the tegument of M. dubius.

Data presented in chapters 3 and 4 indicated that M. dubius accumulates cations and is hyperosmotic to its environment, which could result in an inflow of water. As this does not occur the permeability constant of the body wall was estimated to determine any part it plays in the movement of water. From the permeability constant the activation energy of water penetration may be estimated. If the entry of water is down a concentration gradient the activation energy will be supplied by the gradient and the rate of entry will be unaffected by temperature i.e. the activation energy will be very low. The activation energy of water outflow would be very high with a similarly high temperature coefficient as it must be provided by the metabolic energy of the worm.

At this point two structural aspects of M. dubius should be considered. The first is the absence of an identifiable functional excretory system. King (1965) describes vestiges of an excretory system in M. dubius males and females. No other excretory or osmoregulatory organ has been identified. King comments that the presence of vestiges of a protonephridial excretory system indicates that this species has developed from a form which possessed this functional organ. The total loss of the excretory system is unusual and caused Baer (1961) to write, "En effet, l'absence d'organes excreteurs chez tous les autres Acanthocéphales, laisse supposer qu'il dois exister chez eux un autre mécanisme physiologique qui n'est pas lié à la présence de protonéphridies".

The second structural pecularity of M. dubius is the

lacunar canal system in the tegument. Meyer (1931) examined this canal system in the acanthocephalan Neoechinorhynchus rutili. He described a ramifying system of permanent canals which become progressively more anastomised as they lead away from the main canals. The main canals are lined with a compact meshwork of fibres which become less closely associated with the canals as the latter become smaller in diameter. Meyer considered that the finest canals are the tegumentary fibres themselves. Baer (1961) attribute the function of nutrient circulation to the lacunar canals, and considered that circulation of the fluid on the canals was achieved by turbulence produced by body movements.

Crompton and Lee (1965) examined the lacunar canals of Polymorphus minutus with the electron microscope and described them as thin walled channels. These authors suggested that these canals may connect with the canals seen in the striped layer. (see plate 2.1). Stranack, Woodhouse and Griffin (1966) suggested that the canals of the striped layer may eventually communicate with the canals they observed at the basement membrane of Pomphorhynchus laevis. Nicholas and Mercer (1965) did not report the presence of canals at the basement membrane but reported the apparent formation of vacuoles along the basement membrane. At present it is impossible to determine whether or not these membrane structures are canals or vacuoles.

5.2. Materials and Methods.

5.2.1. The Liquid Scintillation Counter.

Throughout the H_2^3O and C^{14} - U - L - leucine experiments (see chapters 6 and 7) a Beckman CPM 200 liquid scintillation counter was used, except for one experiment described in section 6.3. The liquid scintillation counter, shown in plate 5.1, is used for beta emitting radioisotopes. It estimates the rate of radioactive decay in a sample by counting the number of scintillations of light produced by the scintillation mixture, or "cocktail", in which the radioactive sample is suspended or dissolved. The scintillation cocktail used throughout contained 4 g PPO (2, 5 - diphenyloxazole) and 100 g A.R. naphthalene, dissolved in 1 litre of 1, 4 - dioxan. PPO is the primary fluor, naphthalene is an antiquenching agent and dioxan is the primary solvent. As a beta emitting radioactive nucleus decays an electron is ejected. The kinetic and electrical energy i.e. the ionisation energy, of the electron is transferred to the molecules of the primary solvent, an aromatic hydrocarbon, which become ionised, dissociated or in other ways excited. This excitation energy is transferred among the molecules of the solvent until it is emitted either as an ultraviolet photon or transmitted to the primary fluor which emits the energy as a photon of wavelength 3800 $\mathring{A}$. These photons are detected by the photocathode.

Toluene is among the best primary solvents for liquid

PLATE 5.1.

The Beckman CPM 200 Liquid

Scintillation Counter.


007 - 0000193
Beckman LIQUID SCINTILLATION SYSTEM

scintillation counting as it is highly efficient in the energy transfer process. Toluene is not however miscible with water. For this reason 1, 4 - dioxan has been used as a primary solvent instead of toluene. Dioxan has only 70% the energy transfer efficiency of toluene. Thus naphthalene is included in the cocktail as it increases the efficiency of energy transfer to the primary solute, i.e. it is an anti-quenching agent.

The cocktail also suffers from the deficiency that it absorbs light energy from the atmosphere, which it re-emits as photons producing high initial background counts. This may be overcome by allowing vials containing the samples to stand in the dark, in the sample chamber of the instrument, for 2 hours before counting is commenced.

Any other solute or coloured component in the mixture to be counted with a liquid scintillation counter, will act as "quenching" agent i.e. any material which prevents the production of a photon. Foreign solutes quench chemically, by irreversibly absorbing the excitation energy of the negatron. Coloured materials result in optical quenching by increasing the optical density of the mixture so that the number of photons detected by the photocathode is reduced.

The Beckman CPM 200 liquid scintillation counter is a proportional counter i.e. the output pulse of the photocathode is a linear amplification of the input pulse produced by the photon in the cocktail. The scintillation produced by the fluor is

proportional to the ionisation energy of the negatron. Thus, the output pulse of the photocathode is proportional to the ionisation energy of the negatron. In the counter used, the output pulse is recorded in one of three channels A, B or C depending on its energy and the "isoset" used in each of the channels. The Beckman counter is supplied with 7 isosets which may be plugged into any of the three channels. The isosets instruct the instrument to record fixed energy bands in that channel. For example, if an H^3 isoset is plugged into channel A this channel will record energies above and below the average energy of H^3 (0.006 MeV). The Beckman handbook does not give the width of the energy bands used for the three channels. The isosets, which are located at the right hand side of the control panel, are shown in plate 5.2.

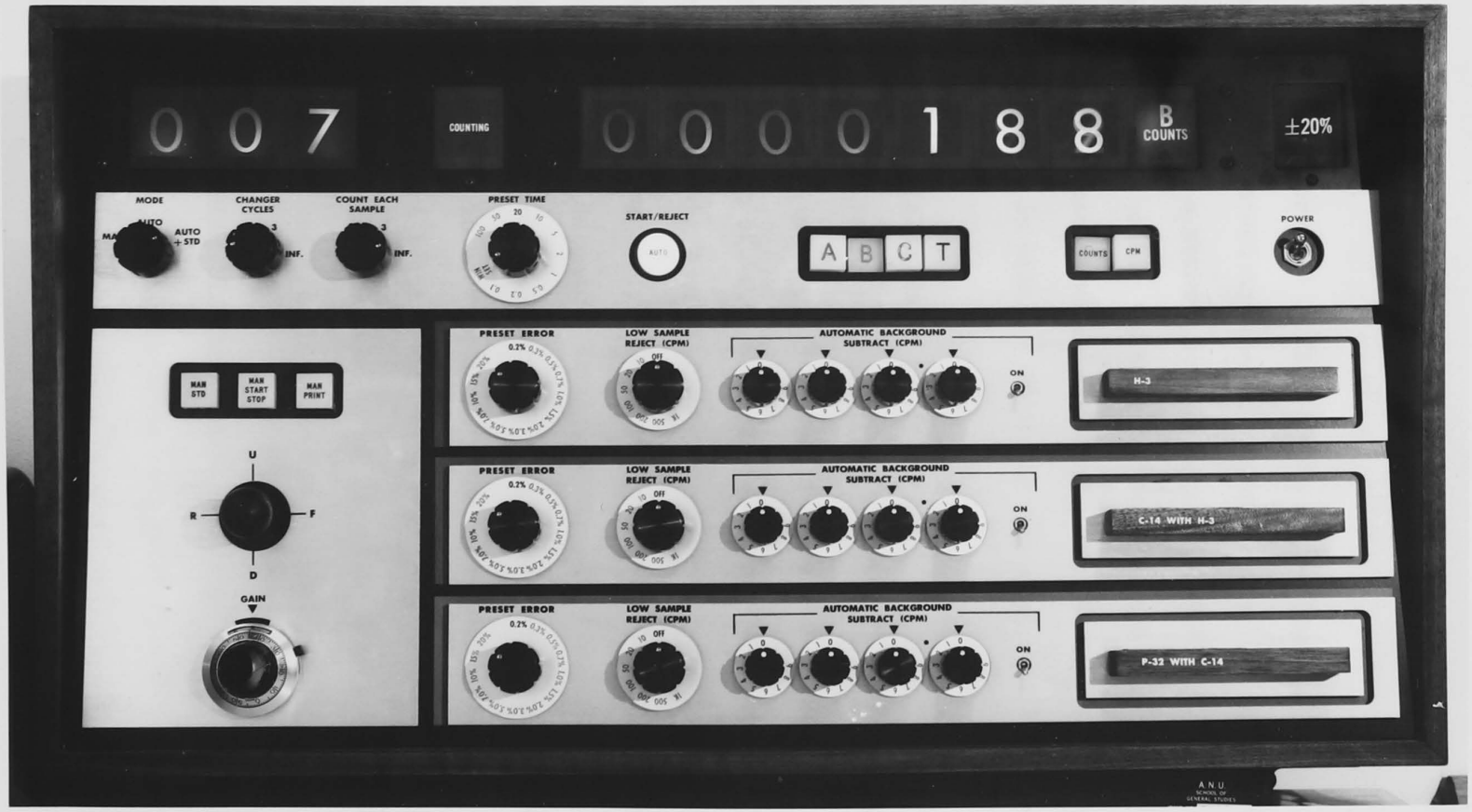
If a quenching agent is present in a count, the first photons to be lost are those of low energy, while those of high energy have their energy reduced. Although the ionisation energy of the negatron is unaltered the constant of the proportionality relating negatron energy to photocathode output signal amplitude is reduced. The energy spectrum of the isotope is therefore modified and the event is recorded at a point lower in the energy scale of that isoset or in the isoset below. If the lowest isoset signals are quenched they may be lost altogether. The gain control on the counter is used to increase the linear amplification of the photocathode input signal so that the output signal amplitude is restored to its unquenched value. The gain setting used for H^3 was

PLATE 5.2.

The Control Panel of the Beckman

CPM 200 Liquid Scintillation

Counter.


007
COUNTING
00001188
B COUNTS
±20%
MODE
AUTO
AUTO + STD
CHANGER CYCLES
INF.
COUNT EACH SAMPLE
INF.
PRESET TIME
START/REJECT
A B C T
COUNTS
CPM
POWER
MAN STD
MAN START STOP
MAN PRINT
U
R
F
D
GAIN
PRESET ERROR
LOW SAMPLE REJECT (CPM)
AUTOMATIC BACKGROUND SUBTRACT (CPM)
ON
H-3
C-14 WITH H-3
P-32 WITH C-14
A.N.U.
SCHOOL OF GENERAL STUDIES

650 and for C^{14} 550.

The counter used is fitted with preset time and error controls. These are set to a maximum counting time (20 minutes was routinely used) and a maximum counting error ($\pm$ 2% was routinely used). The counter prints the accumulated data on a record sheet when one of these conditions is satisfied. The 2% error indicates that the mean shown is within $\pm$ 2% of the true mean with a confidence interval of 95%.

Plate 5.2 also shows the following controls. The Mode control governs the series of operations the counter performs. The manual position activates the 3 control buttons above the Gain Control. The number of times a sample is counted is controlled by the Changer Cycles and Count Each Sample controls. The Low Sample Reject control may be used, to reject samples with less than a preset minimum count. The Automatic Background Subtract (CPM) may be used to deduct a predetermined background from each sample count. The control labelled T, is used to display the time (mins.) a sample has been counting while A B and C display the count of those channels. The Counts and CPM controls display the accumulated count in time T minutes, and this count calculated as cpm respectively.

5.2.2. Sample preparation procedures.

Worms were removed from the rat, weighed, and then transferred separately to 5 ml Hanks balanced salt solution cultures labelled with H_2^3O, and gassed with an atmosphere of 8% CO_2 in 92%

N_2. The worms were cultured for different time intervals and then rinsed by dipping rapidly in three changes of Hanks balanced saline. The female worms were divided into pseudocoel fluid and body wall by cutting off one end of the worm and squeezing the pseudocoel fluid into a tared vessel. The male worms were treated whole. All the components were weighed. Male worms and female body wall were extracted for 24 hours in each of three changes of 2.0 ml of absolute ethanol in 20 ml screw cap glass bottles which were subsequently used in the scintillation counter. Pseudocoel fluids were extracted in 2.0 ml of ethanol for 24 hours each. The components were dried at 105°C for 2 hours and weighed.

A 2.0 ml aliquot of the H_2^3O labelled medium was distilled in a small "Quickfit" still and 0.5 ml of the distillate counted by the procedure outlined below. The H_2^3O was removed from the incubation medium to avoid contamination with inorganic salts which cause severe "quenching" of the count. Fifteen mls of cocktail were added to each extract, and to the distillate from the medium, and the preparations counted.

Background counts were performed on the absolute ethanol used for extracting the isotope, and on the Hanks balanced salt solution used for the incubation media. These counts were deducted from the count of the experimental samples. The calculation methods are shown in appendices 5.1 and 5.2.

5.3. Results.

5.3.1. The first experiment was designed to test whether or not tritium from tritium oxide would penetrate the worm from an incubation medium and its distribution within the worm after various times in culture. The results are shown in table 5.1.

TABLE 5.1. The tritium oxide content of M. dubius and its distribution after various times in Hanks balanced salt solution labelled with tritium oxide.

Worm Component	Incubation Time	H_2^3O Content μl/g worm
Male 1	10 mins.	1.0297
P/coel 2	"	1.2842
B.W. 2	"	1.0115
Male 3	20 mins.	1.5395
P/coel 4	"	1.5745
B.W. 4	"	1.2616
Male 5	30 mins.	1.8031
Male 6	"	1.3589

Two experiments were performed to determine the efficiency of recovering H^3 from the experimental procedures. A standard solution of H_2^3O in distilled water was prepared. A 0.5 ml aliquot was counted. The standard solution was distilled in the same way as the cultures and a 0.5 ml aliquot of the distillate counted. The

ratio of the distilled sample count to the original sample count was 100.2 : 100. Thus it has not been necessary to correct the medium content for the efficiency of recovery.

In order to determine the efficiency of the ethanol extraction procedure, a small piece of dried sponge was soaked in the standard solution used above. This was extracted in ethanol in the same way as the worms and the H^3 content estimated as μl/ml of water absorbed by the sponge. This was compared to the original solution and the recovery was estimated to be 99.7%. Thus the H^3 content of the worms has not been corrected for the efficiency of recovery.

The results show (table 5.1) that tritium oxide does penetrate M. dubius and may be found in both the pseudocoel and body wall after 10, 20 or 30 minutes in labelled culture. The data also show that the average distribution of tritium oxide in the female worms was 56% in the pseudocoel and 44% in the body wall at both 10 and 20 minutes. The distribution within male worms is not known.

5.3.2. The above experiment was repeated with 0.00297 mM of C^{14} - U - L - leucine present in the medium to determine whether any relationship existed between L - leucine uptake and water movement into M. dubius. In order to determine whether any H^3 labelling of the leucine occurred a control culture containing C^{14} - U - L - leucine in Hanks balanced saline labelled with H_2^3O was included.

This mixture was sampled and the remainder of the solution evaporated to dryness at 37°C to remove the H_2^3O. The residue was redissolved in distilled water, sampled and counted. The result showed that the leucine became labelled with H^3 in the medium. This made interpretation of the results impossible.

The C^{14} counts showed that M. dubius was concentrating C^{14} from C^{14} - U - L - leucine to the same extent in both the pseudocoel and body wall, 31 fold over 30 minutes.

5.3.3. A similar experiment was conducted to determine the proportion of water in the worm which was exchanged in vitro. Worms were incubated in Hanks balanced salt solution labelled with tritium oxide. The culture times used were 5, 15, 30, 60, 120 and 240 minutes. Concentration factors were calculated from

$$\frac{\mu l\ H_2^3O/g \text{ worm water}}{\mu l\ H_2^3O/g \text{ (or ml) culture water.}}$$

The results are shown in table 5.2.

TABLE 5.2. The mean proportion of total water (± 1 standard deviation) which was exchanged by male and female M. dubius after various times in Hanks balanced salt solution.

Time (mins)	Female		Whole Male (%)
	Pseudocoel (%)	Body Wall (%)	
5	91.5 ± 7.6	64.7 ± 6.1	84.1 ± 9.8
15	94.7 ± 2.9	70.6 ± 2.9	86.4 ± 5.8
30	93.7 ± 0.4	64.5 ± 16.4	79.6 ± 1.8
60	95.2 ± 2.1	52.6 ± 0.5	82.1 ± 9.5
120	95.8 ± 2.6	72.6 ± 5.9	92.4 ± 1.4
240	90.8 ± 3.7	73.2 ± 0.7	91.8 ± 4.3
Mean	93.4 ± 2.04	66.4 ± 7.73	86.1 ± 5.19

The data in table 5.2 is shown graphically in figure 5.1.

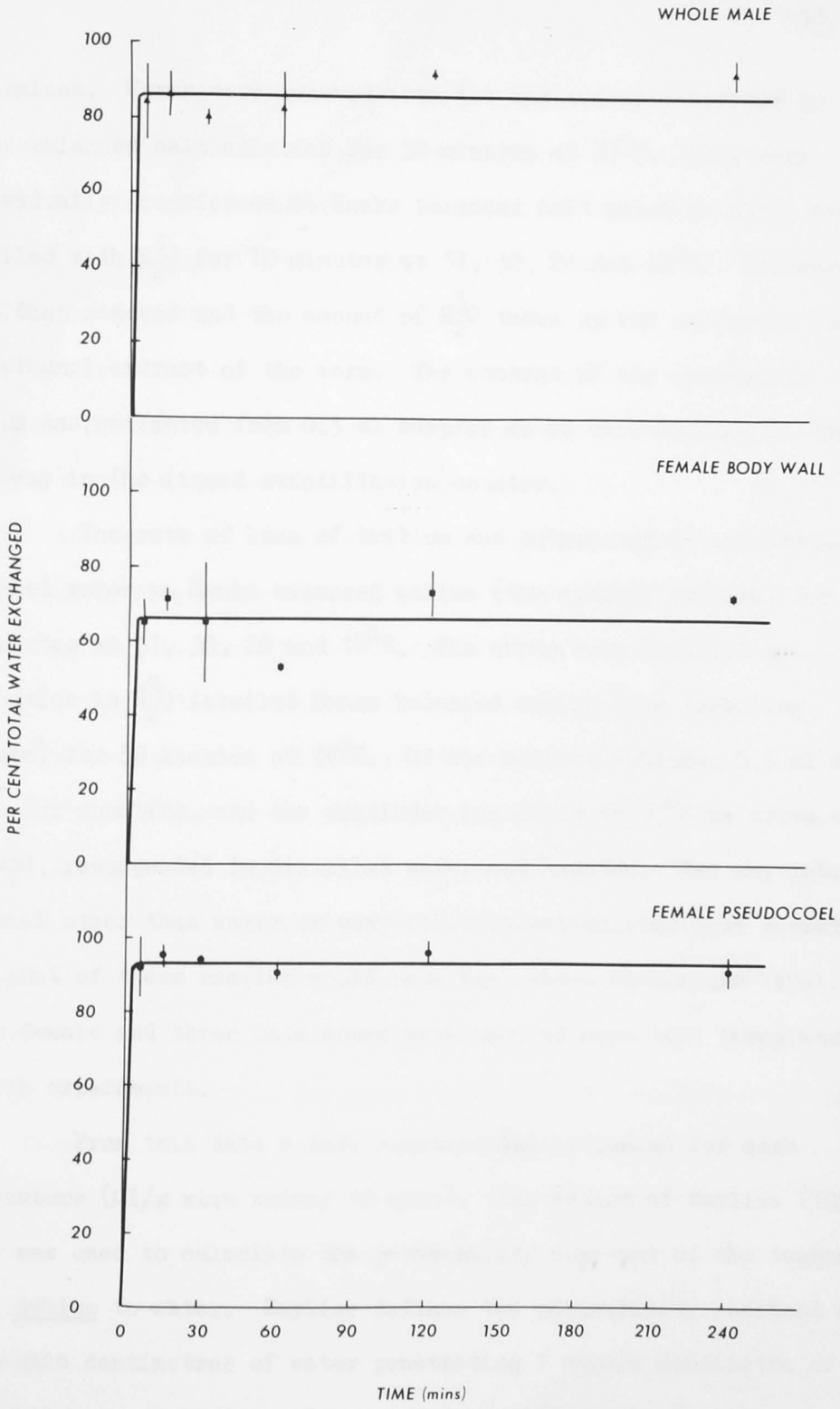
The results show that exchange of water between the worm and Hanks balanced salt solution is very rapid and has reached equilibrium in 5 minutes. Of the tritium oxide present in the female worm at the end of the incubation, 58.8% (± 3.5%) was in the pseudocoel and 41.2% (± 3.5%) was in the body wall. Previous estimates were pseudocoel 56% and body wall 44%.

5.3.4.1. The third experiment attempted to determine the activation energy of water movement into, and out of, M. dubius. The rate at which water enters M. dubius at a number of temperatures was

FIGURE 5.1.

The percentage proportion of total water which was exchanged by M. dubius whole males, female body wall and pseudocoel after various times in Hanks balanced salt solution.

(The lines are drawn by inspection. The vertical line represent $\pm$ 1 standard deviation).


WHOLE MALE
FEMALE BODY WALL
FEMALE PSEUDOCOEL
PER CENT TOTAL WATER EXCHANGED
100
80
60
40
20
0
0
30
60
90
120
150
180
210
240
TIME (mins)

determined. Worms were removed from the rat and equilibrated in Hanks balanced salt solution for 30 minutes at 37°C. They were individually transferred to Hanks balanced salt solution which was labelled with H_2^3O for 10 minutes at 37, 30, 20 and 10°C. The worms were then removed and the amount of H_2^3O taken up was estimated from the ethanol extract of the worm. The content of the incubation medium was estimated from 0.5 ml samples which were counted in the same way in the liquid scintillation counter.

The rate of loss of tritium was determined by incubating labelled worms in Hanks balanced saline (the extract culture) for 10 minutes at 37, 30, 20 and 10°C. The worms were labelled by incubation in H_2^3O labelled Hanks balanced saline (the labelling culture) for 30 minutes at 37°C. Of the extract culture, 0.5 ml was taken for counting, and the remainder was dried at 37°C to drive off the H_2^3O, resuspended in distilled water and counted. Had any labelled material other than water or very volatile metabolites been secreted, the count of these samples would have been above background level. Three female and three male worms were used at each test temperature in both experiments.

From this data a rate constant was estimated for each temperature (μl/g worm water/ 10 mins). The method of Bayliss (1959, 1960) was used to calculate the permeability constant of the tegument of M. dubius to water. Bayliss defines the permeability constant as "... cubic centimetres of water penetrating 1 square centimetre of cell membrane per second under a pressure difference of 1 atmosphere."

Once the permeability constant is known the activation energy of penetration of water may be calculated from the relationship $\log_e (P/\sqrt{T}) \propto \frac{1}{T}$ where P is the permeability constant and T is the absolute temperature (Bayliss 1959, 1960). The method of calculation is given in appendix 5.2.

To determine the permeability constant and activation energy of penetration of water, an estimate of the surface area of the worms used was required. The worms were 8 weeks old. The surface area and body weight of 7 and 8 week old worms were determined by weighing worms taken from the rat gut and then stretching them with a 10.0 gram weight. While stretched the length and outside diameter were measured with a vernier calliper. The worms were treated as a cylinder, and the surface area calculated from π x d x l where d is the diameter and l is the length. The formula surface area (cm^2) = body weight (gram) $^{2/3}$ x A was used to calculate the value of the constant A. The values obtained were for females, A = 19.07 $\pm$ 2.13 and for males A = 15.32 $\pm$ 2.19. These factors were then used to calculate the surface area of the worms used in this experiment. The rate constants (mean μl/gram worm water/10 minutes) and the mean permeability constants calculated are shown in table 5.3 (see also appendices 5.1 and 5.2).

TABLE 5.3. The mean rate constants of water uptake (μl/g worm water/10 minutes) and the permeability constants estimated according to Bayliss (1959, 1960).

Sex	Temperature (^{o}K)	Mean Rate Constant μl/g/10 mins.	Mean permeability constant (ml/cm^2/sec/atm)
Female	310	782 $\pm$ 55.4	$0.583 \times 10^{-5} \pm 0.044 \times 10^{-5}$
	303	774 $\pm$ 43.8	$0.606 \times 10^{-5} \pm 0.031 \times 10^{-5}$
	293	651 $\pm$ 67.2	$0.525 \times 10^{-5} \pm 0.029 \times 10^{-5}$
	283	665 $\pm$ 21.4	$0.496 \times 10^{-5} \pm 0.050 \times 10^{-5}$
	Mean	-	$0.552 \times 10^{-5} \pm 0.054 \times 10^{-5}$
Male	310	847 $\pm$ 30.6	$0.259 \times 10^{-5} \pm 0.024 \times 10^{-5}$
	303	824 $\pm$ 37.3	$0.260 \times 10^{-5} \pm 0.028 \times 10^{-5}$
	293	781 $\pm$ 9.4	$0.263 \times 10^{-5} \pm 0.0001 \times 10^{-5}$
	283	704 $\pm$ 27.1	$0.222 \times 10^{-5} \pm 0.008 \times 10^{-5}$
	Mean	-	$0.251 \times 10^{-5} \pm 0.024 \times 10^{-5}$

E_A is the Arrhenius or apparent activation energy which is calculated from the slope of the straight line, obtained by plotting $\log_e$ k (the rate constant) against the reciprocal of the absolute temperature; and R the gas constant, i.e. E_A = slope x 1.9871. As the data was plotted as $\log_{10}$ k, the expression becomes 2.303 x 1.9871 x slope. The activation energies calculated in this way were 8,840 cal/mole for females and 9,300 cal/mole for males.

The apparent Q_{10} of reactions with those apparent activation energies is given by $Q_{10} = e^{\left(\frac{10 \times E}{R \times T\ (T + 10)}\right)}$ (Kruyt and Overbeek (1960)); for E = 8,840 cal/mole, Q_{10} (27 - 37^{o}C) is 1.61

and for E = 9,300, Q_{10} (27 - 37°C) is 1.65. Bayliss (1959) states that purely physical processes generally have a low Q_{10}. Some chemical processes show a high Q_{10} but this is not true of all chemical processes. As this data is drawn from a whole animal the Q_{10}'s calculated above represent the temperature dependance of the entire penetration process. Further the penetration of 56% of this water involves traversing 2 membranes as approximately this proportion of the water enters the pseudocoel.

5.3.4.2. A sample of the extract culture was dried at 37°C and resuspended in distilled water. This sample was counted and the result compared with a sample of Hanks balanced salt solution treated in the same way. The mean counts which resulted were 52 cpm for the dried extract culture and 50 cpm for the Hanks balanced saline control. Thus it was concluded that the tritium had been present as water or as some other volatile product.

The water loss section of this experiment measured the rate of water passage out of M. dubius, by estimating the tritiated water content of the extract culture medium after incubating H_2^3O labelled M. dubius for 10 minutes. The initial content of label in the worms after incubation in the labelling culture was estimated from the content (μls) of H_2^3O in the extract culture plus the content (μls) of H_2^3O remaining in the worm (see appendix 5.3). In this way the mean per cent loss of labelled water at 37, 30, 20 and 10°C was calculated. The results are shown in table 5.4.

TABLE 5.4. The mean per cent loss of tritium labelled water ($\pm$ 1 standard deviation) from labelled male and female M. dubius after 10 minutes in vitro culture in Hanks balanced saline at 37, 30, 20 and 10°C.

	No. of worms	TEMPERATURE (°C) 37	30	20	10
Female	3	90.0 $\pm$ 2.7	87.4 $\pm$ 5.1	80.1 $\pm$ 2.2	76.6 $\pm$ 6.1
Male	3	94.8 $\pm$ 3.6	94.3 $\pm$ 2.1	87.5 $\pm$ 3.7	80.7 $\pm$ 6.6

From this data it can be seen that up to 95% of the H_2^3O assimilated by M. dubius is lost in 10 minutes in Hanks balanced saline at 37°C. The reduction in the rate of loss over the temperature range is $\frac{90.0 - 76.6}{90.0} \times \frac{100}{1} = 14.8\%$ for females and $\frac{94.8 - 80.7}{94.8} \times \frac{100}{1} = 14.8\%$ for males. The reduction in uptake rate is $\frac{782 - 665}{782} \times \frac{100}{1} = 14.9\%$ for females and $\frac{847 - 704}{847} \times \frac{100}{1} = 16.8\%$ for males (see table 5.3). Thus reducing the temperature from 37°C (310°K) to 10°C (283°K) has affected equally the inward and outward movement of water.

This result demonstrates that the body fluids of M. dubius are isotonic with Hanks balanced salt solution, and is in agreement with the finding in chapter 3 and 4 that the readily exchangeable Na and K produce isotonicity with Hanks balanced saline. Thus the non-exchangeable or slowly exchangeable cations must be osmotically

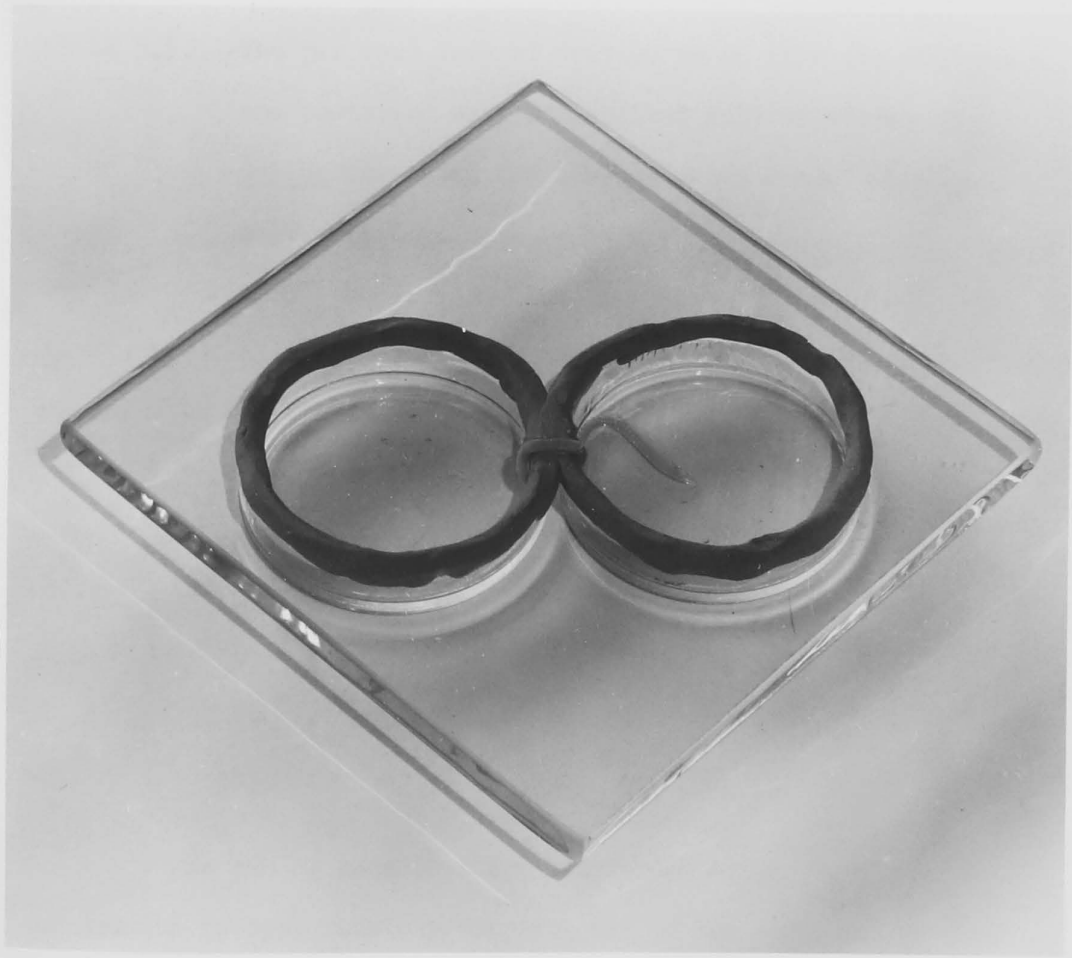
inactive. The regions shown in figure 3.4 may be responsible if the cations are tightly bound. The finding that 34% of the female body wall water was non-exchangeable or exchanged only slowly may indicate that this water is associated with the slowly exchangeable cations.

5.3.5. The results of the previous experiment provided estimates of the activation energy of water movements. Two experiments were then performed to determine the site of water exchange. The apparatus shown in plate 5.3 was used throughout. The apparatus consists of two small petri dishes with a rim of modelling clay placed around the lip at the top. The test solutions are placed in the dishes and the worm is placed over the lip of the dishes so that one end is in each solution. The dishes were then covered with a sheet of $\frac{1}{4}$" plate glass to prevent evaporation and capillary creep of fluids along the outside of the worm. A test with a worm killed by boiling showed that radioactivity was not transferred from one vessel to the other.

In the first of these experiments worms were labelled in Hanks balanced salt solution for 30 minutes and then transferred to the test apparatus in the following way. Three females and three males were placed in individual sets of apparatus so that half the worm lay in each vessel. The worms were incubated in this state for 15 minutes. 0.5 ml samples of medium from the vessels containing the anterior and posterior halves of the worm were then counted.

PLATE 5.3.

The apparatus used to determine the site of water exchange in M. dubius (see text for description).



This process was repeated with the anterior quarter of the worm in one vessel and posterior three quarters in the other, and vice versa. The results are shown in table 5.5..

TABLE 5.5.. The per cent loss of tritium oxide from the anterior and posterior ends of labelled male and female M. dubius incubated for 15 minutes in Hanks balanced salt solution.

Sex		Ant. $\frac{1}{2}$: Post. $\frac{1}{2}$	Ant. $\frac{1}{4}$: Post. $\frac{3}{4}$	Ant. $\frac{3}{4}$: Post $\frac{1}{4}$
	1	43 : 57	34 : 66	67 : 33
Female	2	42 : 58	23 : 77	65 : 35
	3	47 : 53	24 : 76	84 : 16
Av.		44 : 56	27 : 73	72 : 28
	4	54 : 46	44 : 56	39 : 61
Male	5	63 : 37	41 : 59	61 : 39
	6	50 : 50	50 : 50	38 : 62
Av.		55 : 45	45 : 55	46 : 54

It can be seen that for the female worms the per cent loss of tritium is approximately proportional to the length of worm in each vessel. For males this relationship does not hold true but this may be attributed to the difficulty of arranging the shorter male worms in the apparatus.

The second experiment investigated the site of uptake by placing the anterior end of three female and three male worms in one vessel which contained Hanks balanced saline labelled with tritiated

water, and the posterior end in unlabelled Hanks balanced saline. The worms were incubated in individual sets of apparatus for 20 minutes, and the previously unlabelled Hanks balanced saline was counted for radioactivity. The process was then reversed by placing the posterior end in the labelled medium. This experiment was designed to determine whether or not a particular site on the worm was responsible for water uptake. The results are shown in table 5.6..

TABLE 5.6.. The transfer of tritium oxide by M. dubius from labelled to unlabelled Hanks balanced salt solution (background deducted) after 20 minutes in vitro incubation.

Sex		End labelled	Count cpm	End tested	Count cpm
Female	1	Ant.	30,807	Post.	1,029
	2	"	"	"	61
	3	Post.	"	Ant.	49
	4	"	"	"	71
Male	1	Ant.	30,807	Post.	50
	2	"	"	"	57
	3	"	"	"	76
	4	Post.	"	Ant.	85
	5	"	"	"	122
	6	"	"	"	195

The amount of tritium oxide transferred is very small. This may reflect the importance of the pseudocoel in transferring tritium oxide along the body wall under these conditions. In the apparatus the pseudocoel was compressed between the glass sealing plate and the top of the petri dishes. If the pseudocoel was occluded, and transfer along the worm takes place by this route, only a small transfer would be expected. These results demonstrate that water may enter and leave M. dubius at any point along the body wall.

An incidental observation from these experiments was that after 20 minutes in the apparatus, the phenol red indicator of the Hanks balanced saline surrounding the worm was yellow. This indicates that the worm is excreting acid products from its entire body surface but this is only seen when the worm is restrained in a small volume of Hanks balanced saline. Whether or not the excretion of acid metabolites is associated with the rapid exchange of water, or if the two materials move by the same path, is not known.

5.4. Discussion.

The data presented in chapter 5 show that both male and female M. dubius continuously exchange their water with Hanks balanced salt solution in vitro incubation medium. It is not known whether or not the same continuous exchange occurs in the rat gut, but it appears most likely that it would continue to occur. The exchange of water between M. dubius and its medium in vitro is extremely rapid, and indicates that M. dubius maintains a rapid

exchange of a fixed proportion of its water with its environment. The pseudocoel rapidly exchanges its water with either the body wall or the environment via the body wall, whereas it does not readily exchange its cations.

The activation energy of water penetration, from 191 mM NaCl solution, into Bufo melanostictus is 9,000 cal/mole (Dicker and Elliott (1967)). This figure for a toad is remarkably close to the estimate for M. dubius. The authors cite Wang (1965), who gives the activation energy of self diffusion of water as 5,000 cal/mole. Thus in M. dubius, as in B. melanostictus, the rate of water entry is not diffusion limited. Dicker and Elliott argue that the uptake of Na was responsible for the reduction in activation energy of penetration of water from 12,000 cal/mole in pure water to 9,000 cal/mole in 191 mM NaCl. They point out that the reduction of 3,000 cal/mole is supplied by the energy of the Na pump. They cite Zerahn (1956, 1961) who estimated the activation energy of uptake of Na by amphibia as 2,000 - 6,000 cal/mole with the Na ion carrying 1.7 - 4 water molecules in its hydration shell (Hasted, Ritson and Collie (1948), Robinson and Stokes (1959)). Thus if an activation energy of 6,000 cal/mole for Na ion is accepted and the hydration is 2 molecules, then the energy input from the Na pump is 3,000 cal/mole of water, which agrees with the reduction in activation energy given above by Dicker and Elliott (1967).

Whether or not the activation energy of water entry into M. dubius is supplemented by the activity of the cation pumps is

not known. It has however been determined that the activation energy of water penetration into female M. dubius is 8,840 cal/mole, slightly less than the 9,300 cal/mole estimate for male M. dubius.

Bayliss (1960) gives a list of permeability constants for various tissues. The constants range from 6,000 x 10^{-6} ml/cm^2/sec/atm for the glomerulus of the frog kidney to 0.06 x 10^{-6} ml/cm^2/sec/atm for Amoeba proteus in fresh water. The permeability constant of soft tissues from a number of vertebrate animals range from 0.5 x 10^{-6} to 4.2 x 10^{-6} ml/cm^2/sec/atm. Fibroblasts from tissue cultures of mouse, rat and chick range from 0.65 x 10^{-6} to 1.70 x 10^{-6} ml/cm^2/sec/atm. The mean values estimated for the permeability constant of the tegument of male and female M. dubius (0.251 x 10^{-5} and 0.552 x 10^{-5} ml/cm^2/sec/atm respectively) are slightly higher than the representative values for vertebrate soft tissue fibroblasts.

The semipermeability of the basement membrane is demonstrated by the exchange of 93% of pseudocoel water without any appreciable exchange of cations, determined from Na^{24} and K^{42} studies (chapter 4). The remaining 7% of the water in the pseudocoel may be associated with the eggs. Potts and Parry (1964) discussed the methods by which almost complete impermeability may be achieved by cell walls but point out that a path of exchange between the cell and its environment is essential for respiratory, assimilatory and excretory exchanges. These authors discuss superficial coats on cells, such as chitin, keratin and mucus

layers which reduce their permeability. von Brand (1940) detected a chitin layer on the inner surface of acanthocephalan eggs, but did not detect any other chitinous structures in the animal. West (1964) described a "fibillar coat" which was a membrane structure around the acanthor of 2 acanthocephalans parasitic in fish. Thus the eggs of the Acanthocephala could be impermeable to water. The 33.6% non-exchangeable water in the body wall may be contained within an impermeable membrane, but this seems unlikely.

It could, however, be bound to proteins. In order to determine whether or not this was possible, the amount of water the proteins would have to bind was calculated as follows:-

The mean non-exchangeable water in the body wall was 33.6% $\pm$ 7.7% of the total water. The mean weight of non-exchangeable water in the body wall of the worms was estimated to be 100.3 $\pm$ 5.51 g H_2O for each 100 g of dried body wall, i.e. there is the same weight of non-exchangeable water in the body wall as there is structural, ethanol insoluble, non-volatile material. It is known from data accumulated for chapter 6 that the nitrogen content of the dry body wall weight of M. dubius is 7.11% $\pm$ 1.33%. Thus proteins represent approximately 7.11 x 6.25 = 44.4% of the dry body wall weight (6.25 is the figure given by Diem (1962) to convert protein nitrogen to protein). Thus if the proteins were binding the non-exchangeable water they would require to bind $\frac{100.3 \times 100}{44.4}$ = 226% of their own weight. This may be an overestimate because the proportion of non-exchangeable water has been estimated to be

as low as 25.9%, i.e. 33.6% - 7.7%. Bull (1964) gives a table of saturation binding of water by a group of materials. These estimates range from 8.7% for stretched nylon to 85% for gelatine. It seems unlikely that any fibrous or lattice structure could effectively bind 226% of its own weight. Bayliss (1959) suggests that the most frequent estimate of bound water by proteins is 20 - 50%. In the body wall of M. dubius, therefore, it appears that the proteins alone are not responsible for binding the non-exchangeable water.

Some of the non-exchangeable water may be bound to the non-exchangeable cations. The following calculation gives a rough idea of the proportion of non-exchangeable water which could be associated with the non-exchangeable cations and for which several questionable assumptions must be made. In the female body wall 74% of the Na and 28% of the K had not exchanged after 4 hours in Hanks balanced salt solution. For the purposes of this calculation it is assumed that this represents the equilibrium position. The total initial female body wall Na content was 6.42×10^{-4} g Na/g H_2O or 642 μg Na/g H_2O, and the total initial K content 62.05×10^{-4} g K/g H_2O or 6205 μg K/g H_2O. This is a minimum estimate as the cation content increases in Hanks balanced saline. Thus the non-exchangeable Na and K content is $\frac{74 \times 642}{100}$ = 475 μg Na/g H_2O = approximately 21 μEq Na/g H_2O and $\frac{28 \times 6205}{100}$ = 1737 μg K/g H_2O = approximately 44.5 μEq K/g H_2O.

If the hydration shell of Na and K is taken as 2 molecules

(Dicker and Elliott (1967)) there will be 2 x(21 + 44.5) = 131 μM H_2O/g H_2O which is bound to the cations. This is an underestimate because K, which is a smaller ion than Na, may have a larger hydration shell because of its greater electrostatic charge density. 131 μM H_2O/g H_2O represents 18 x 131 = 2358 μg H_2O/g H_2O. Thus $2358 \times 10^{-6} \times 10^{2} = 0.2358\%$ of the total water could be bound to the cations. The total non-exchangeable water is 33.6%. Thus it can be seen that the hydration shell of the non-exchangeable cations could not account for any significant proportion (0.7%) of the total non-exchangeable water.

Glynn (1959a) suggests a maximum figure of 16% for non-exchangeable cell water and points out that if the intracellular K is bound, a higher proportion ofcell water would require to be bound to maintain osmotic equilibrium. Results in chapter 4 suggest that some of the body wall K is bound, as 28% of it had not exchanged in Hanks balanced saline after 4 hours. Thus an estimate of 33.6% for bound water is not unreasonable, but the means by which it is bound is not known.

The overall isotonicity of Hanks balanced salt solution and the hyperosmotic freezing point depression estimates of the body fluids cannot be reconciled unless the overall high osmotic pressure produced by areas with a very high cation content which are separated from the bulk of the internal environment. The cations within M. dubius may be in part bound to proteins. However, it seems unlikely because the calculated cation osmotic pressures form the

bulk of the osmotic pressure calculated from freezing point depression measurements. The total osmotic pressure calculated from freezing point depression would be a minimum estimate as volatile components would be lost during preparation of the samples. It would therefore appear that the high local osmotic pressure is produced by high local concentrations of cations either enclosed in impermeable regions within the body wall or tightly bound so that they are osmotically inactive. The non-exchangeable water may be localised in the same area or areas.

The vacuoles observed below the striped layer and above the basement membrane of M. dubius as well as the lacunar canals are of unknown function. It would seem unlikely that the vacuoles would be responsible for the retention of the non-exchangeable or slowly exchangeable Na, K and water. If the vacuoles contain the materials, and are moving, this must result in a change in the distribution of the contents; and the concept of a stationary vacuole is difficult to accept. The lacunar canals may provide this protected environment but insufficient is known about their walls to reach any conclusion. Nicholas (personal communication) has experienced difficulty in dehydrating and clearing the lacunar canals of M. dubius prepared for whole histological mounts. This indicates that the dehydrating agent, (ethanol) has failed to penetrate the lacunar canals. They could therefore provide a reservoir of non-exchangeable water.

CHAPTER 6

THE ASSIMILATION OF NUTRIENTS BY M. DUBIUS (ACANTHOCEPHALA) WITH SPECIAL REFERENCE TO THE AMINO ACID L - LEUCINE.

6.1. Introduction.

The numerous papers on active transport which have been published in the last few years have not been confined to the transport of cations. Transport systems have been described for the accumulation of both amino acids and carbohydrates. Many of these reports have concerned the absorption of materials by the small intestine of mammalian vertebrates, in particular of the rat and the hamster. More recently, reports of transport systems in the absorptive surfaces of various helminthes, including Acanthocephala, have appeared. The remaining chapters of this thesis describe investigations into the absorptive capabilities of M. dubius under various in vitro conditions.

To reiterate, its main characteristic, active transport is the transport of a material against an electrochemical gradient. Rosenberg (1954) gives a series of factors which may be used to indicate the presence of an active transport system. The first of these is the effect of concentration. If the movement of a solute is due to active transport rather than diffusion, the rate of transport will not be a linear function of the concentration

gradient, and saturation phenomona may occur at high concentrations. A second indication of active transport is competition between chemically similar substances, although this will, of course, depend on the relationship between the two substances and the specificity of the transport system. Similar inhibition of the transport mechanism may be obtained by slight structural alteration of the transported molecule. Again, this will depend on the nature of the alteration and whether or not it interferes with the reactive site of the molecule to be transported. The temperature coefficient (Q_{10}) may be unusually high, and may approximate to that of enzymatic reactions. Transport is termed active because it is dependent on a supply of energy from the metabolic processes of the cell. Thus transport may be inhibited by the absence of oxygen or the inhibition of the energy metabolism of the cell, but Rosenberg (1954) stresses that these factors may influence the transport mechanism itself or the membrane structure, or other ancillary structures and reactions, thereby inhibiting the transport mechanism indirectly.

Danielli (1954b) discussed the nature of the carrier molecule. He pointed out that to transport through the plasmalemma the carrier molecule must be readily soluble in lipid and insoluble in water. It must not, therefore, readily form hydrogen bonds with water molecules, but must rapidly and reversibly form a complex with the molecule to be transported. The complex must also be hydrophobic, readily soluble in lipid and must diffuse, or in some other way translocate, through the lipid moiety of the membrane.

Smyth (1964) discussed the transport of amino acids from the intestinal lumen into the blood - stream. He states that amino acids and dipeptides are transported, but no larger units. Gitler and Martinez - Rojas (1964) concluded that protein nitrogen is absorbed from the intestine in the form of amino acids. Smyth (1964) concluded that the observed competition between some amino acids arose from their common preference for one transport site or carrier; disproportionate competition between methionine, proline and glycine indicated the presence of two carriers, one transporting all three amino acids and the other only glycine and proline. This evidence, plus the demonstration that the transport processes followed Michaelis - Menten kinetics, showed that a carrier represents a rate limiting step, and that many carriers may be present in an active absorptive surface such as the intestine.

Quastel (1960) reviewed the work on active transport of amino acids and sugars by the intestine of various animals. He gives a list of amino acids which are transported by everted sacs of hamster intestine. The list includes proline, threonine, alanine, glycine, serine, valine, histidine, hydroxyproline, phenylalanine, isoleucine and leucine. Cysteine and the amides glutamine and asparagine are actively accumulated by the rat gut. Wiseman (1953, 1955) showed that L but not D isomers of alanine, phenylalanine, methionine, histidine and isoleucine were actively transported by hamster intestine, and also suggested that transport of amino acids was restricted to mono-amino mono-carboxylic amino acids. Lin, Hagihira

and Wilson (1962) demonstrated that two further transport systems existed in the hamster intestine. They found that basic amino acids were actively accumulated and that the proline transport site would also transport sarcosine, N, N - dimethylglycine, and betaine. They confirmed the finding that D - isomers of many amino acids were not transported, and determined the molecular architecture, of the molecule to be transported, necessary for active transport to occur. They showed that active transport of amino acids require that the carboxyl group remains free and that it cannot be replaced by another negatively charged group. In addition the amino group can not be attached to the β carbon atom nor could it be substituted; neither can the ∝ hydrogen be replaced. From this evidence it was concluded that the carrier distinguished the D and L forms of the amino acids by the interaction with the ∝ hydrogen, amino and carboxyl groups. Thus, the amino acid carriers are relatively specific for molecular architecture.

Newey (1967) reviewed the literature on the transport of carbohydrates by the intestine and determined that glucose and galactose were absorbed faster than fructose, which was absorbed faster than mannose. Wilson (1963) determined that the minimum structural requirement for the active transport of a sugar was a pyranose ring with a carbon atom attached to C - 5 and a hydroxyl group attached to C - 2 in the same configuration as D - glucose. The necessity for this configuration has been challenged by the demonstrations that xylose, which does not have a C atom attached to

the C - 5 of the pyranose ring, and D - mannose, which lacks the hydroxyl group were transported, (Newey (1967)).

Quastel (1960) pointed out that glucose was absorbed from the intestine in two ways. That part of the glucose uptake was by passive diffusion but the majority was by active transport was shown by the observations that the process was inhibited by anoxia or 2, 4 - dinitrophenol, that it displayed Michaelis - Menten kinetics, and that transport took place against a concentration gradient.

The nature of the carrier molecules or mechanisms has not been determined, but several workers indicate that cations are involved in the systems which transport amino acids and carbohydrates. Ricklis and Quastel (1958) demonstrated that glucose was not transported by isolated guinea pig intestine if K and Mg ions were not present simultaneously in the intestinal lumen.

Crane, Miller and Bihler (1960) demonstrated that Na ion was required for active transport of sugars by hamster intestine. If the Na ion was not present, or if the cardiac glycoside strophanthidin was present, the accumulation of sugars against a concentration gradient did not occur. They also showed that passive movement of sugars required the presence of Na ions but was not affected by the presence of strophanthidin. These workers further demonstrated that transported sugars accumulated rapidly and attained an equilibrium across the gut epithelium. The level and rate of attainment of this steady state depended directly on the

external Na ion concentration. The level of the steady state attained in an isotonic solution (143 mM Na) was increased if the solution was made hypertonic by increasing the Na ion concentration to 180 mM.

Crane, Forstner and Eichholz (1965) also demonstrated that active sugar transport depended on the presence of Na ion. They published a series of K_m (Michaelis - Menten Constant) values for the transport of glucose by hamster intestine in the presence of various concentrations of Na ions. The results showed an increasing K_m as Na ion concentration decreased. At 145 mM Na, K_m = 4 mM glucose, at 48 mM Na, K_m = 15 mM glucose, 24 mM Na, K_m = 40 mM glucose and 0 mM Na, K_m = 100 mM glucose. They discussed this finding in relation to the cation balance of cells and tissue fluids. They pointed out that the Na ion concentration within cells was lower than in tissue fluids and in the gut lumen, resulting in a higher K_m for the outward transport of glucose. Thus the transport equilibrium favoured the inward transport of glucose. This conclusion was supported by the evidence of Crane (1964) that if the normal cation equilibrium was reversed and the cell interior contained more Na ion than the tissue fluid, active outward transport of glucose resulted.

Kuchler (1967) studied the effects of various Na and K ion levels on the accumulation of amino acids, protein synthesis and cell growth of LM - strain mouse fibroblasts in tissue culture. He showed that the ratio of K to Na directly governed the volume of

the cells. The volume increased as the K to Na ratio increased, the increase in volume being directly related to the increase in intracellular K. He also showed that the simultaneous presence of Na and K ions was required for the cells to accumulate leucine. The most favourable ratio was 140 mM Na to 10 mM K. Other cations apparently exerted no effect. On the basis of this data Kuchler proposed two alternative hypotheses. Firstly, that the Na and K pumps established a concentration gradient and that the amino acids were then accumulated by exchange diffusion between K ion and the amino acid. Alternatively there was a linked entry of Na and amino acid, with the energy being provided by the diffusion of the Na ion down its electrochemical gradient, which was established by the cation pumps. He estimated that two Na ions were transported for one glycine molecule. He demonstrated inhibition of amino acid transport by ouabain, which strengthens the suggestion that the cation pumps are directly involved in the transport of amino acids. Kuchler's (1967) second hypothesis proposed a mechanism whereby the transport of Na and K was linked in a reaction that activated the carrier molecule.

There is therefore strong evidence for the existence of specific carriers in intestinal and other tissues for the active transport of amino acids and carbohydrates. Several investigations along similar lines have been undertaken on cestodes and Acanthocephala. Daugherty (1957) demonstrated that temperature exerted a greater effect on the accumulation of methionine and

cystine by Hymenolepis diminuta (Cestoda) than could be explained on the basis of passive penetration. He also determined that inhibitory effects were exerted between amino acids. Senturia (1964) also demonstrated active accumulation of methionine by H. citelli. He examined the kinetics of this process and showed that saturation phenomena occurred. Competitive inhibition between amino acids was also observed. Hopkins and Callow (1964) demonstrated that methionine was actively accumulated by H. diminuta, and that 50% of the accumulated material was lost within one hour in in vitro media. The same rapid exchange was demonstrated in vivo showing that amino acid exchange between host and parasite may be a dynamic process. The findings given above are further complicated by the work of Goodchild and Wells (1957) who examined the amino acids in the rat gut and H. diminuta. They found that all the amino acids present in the gut were present in the parasite with the exception of methionine. It is difficult to understand how this amino acid could be missing when it is actively transported. It may be that in vitro culture media induce radical changes in the normal assimilatory mechanisms of the parasite, or perhaps that the concentration of the methionine was below the level of resolution of the techniques employed by these workers.

Read, Simmons and Rothman (1960) and Read and Simmons (1962) demonstrated the active transport of amino acids by Calliobothrium verticillatum, a cestode from the spiral valve of the dogfish. They discussed pH in relationship to speed of

penetration of L - leucine. Maximum penetration of this amino acid was attained at a pH of 7.4.

Read, Rothman and Simmons (1963) in their exhaustive study of amino acid accumulation by H. diminuta demonstrated convincingly that amino acids are actively transported into this tapeworm. They suggested the existence of four loci for the transport of amino acids. The first locus was described as the arginine/lysine locus. The second was for the transport of phenylalanine and tyrosine, while the third was specific for monoaminodicarboxylic amino acids. The final locus was that for the transport of the neutral amino acids. They were unable to demonstrate any effect on the rate of uptake of methionine by varying Na:K ratios, or including Strophanthin G in the culture medium.

Phifer (1960) studied glucose uptake by H. diminuta and concluded that the process was active as it showed a high Q_{10}, could be inhibited by metabolic poisons and followed saturation kinetics. He showed that the rate of penetration of glucose into H. diminuta was not dependant on the presence of the K ion.

Read, Rothman and Simmons (1963) applied Michaelis-Menten kinetics to describe the characteristics of amino acid uptake by H. diminuta (Cestoda). They calculated the K_t and V_{max} of the transport mechanism from a reciprocal plot of the rate of transport against amino acid concentration, i.e. 1/v against 1/(s). They repeated the work including a second amino acid in the medium. They showed that active accumulation of an amino acid could be

competitively inhibited by the presence of a second, or more, amino acids, if they all shared the same transport locus. From the Michaelis - Menten analysis of a series of experiments they estimated a series of inhibitor constants for the reduction in the acquisition of one amino acid brought about by the presence of another. They then derived an equation to forecast the uptake of a single amino acid from a mixture of amino acids. The equation is:

$$v = \frac{V_{max}}{\frac{(K_t)}{(S)} + 1 + \frac{(K_t)\ (S)}{(K_{t1})\ (S)} + \frac{(K_t)\ (S^1)}{(K_{t2})\ (S)} + \text{---------} \frac{(K_t)\ (S^n)}{(K_{tn})\ (S)}}$$

where v is the predicted uptake; (S) is the culture concentration of the amino acid whose uptake is to be predicted; K_t is the Michaelis - Menten constant for S; K_{tn} is the Michaelis - Menten constant for S_n. Thus with a knowledge of the Michaelis - Menten constants and concentrations of all the amino acids in the mixture, the uptake of any one of them may be predicted.

Bjorkman and Thorsell (1964) examined the cuticle of F. hepatica and describe a surface which resembles the micro - villi of the intestine. In a subsequent paper these workers reported that amino acids would penetrate the cuticle of F. hepatica, but ferritin, a molecule of high molecular weight, and a protein would not. This result suggested that absorption through the cuticle was not pinocytotic, but that the surface membranes were capable of allowing the passage of materials without disrupting their continuity. (Thorsell and Bjorkman (1965)).

Edmonds (1965) examined the uptake of C^{14} - labelled leucine by M. dubius from in vitro culture media. He did not examine the kinetics of the process but showed that the presence of DL - valine, DL - serine, or DL - methionine reduced the assimilation of L - leucine. Edmonds and Dixon (1966) incubated M. dubius in vitro and showed that the worm would ingest carbon particles with an average diameter of 250 Å and particles of thorium dioxide with average diameters ranging from 70 to 250 Å. Personal communication with Dr. Edmonds revealed that these particles were only ever seen at the surface layers of the body wall, which suggested that they had only penetrated the canals observed at the surface of the tegument, the striped layer.

Rothman and Fisher (1964) investigated the assimilation of amino acids by M. dubius, and showed that methionine was concentrated over a period of one hour. They went on to show that the assimilation of five different amino acids followed Michaelis - Menten kinetics, and that mutual competitive inhibition occurred. They tested the hypothesis proposed by Read, Rothman and Simmons (1963) that the inhibition of uptake of an amino acid can be predicted from the concentrations of substrate and inhibitor and the relevant Michaelis - Menten constants. They concluded that the inhibited uptake could be predicted in this way.

Graff (1964), working on the incorporation and metabolism of C^{14} labelled glucose by M. dubius, found that the total glucose absorbed by M. dubius varies with the surface area to volume ratio

of the worms. This is evidence that glucose is assimilated and metabolised, but it is not known if a transport system is involved. The evidence that the uptake depends on surface area may indicate that the bounding membrane is involved.

In reviewing the uptake of nutrients by parasitic helminths attention has been restricted to those lacking an alimentary canal and therefore faced with a physiological situation comparable to M. dubius.

6.2. Materials and Methods.

6.2.1. Rothman and Fisher (1964) showed that M. dubius could accumulate the amino acids L - leucine, L - isoleucine, L - serine and L - alanine. An experiment was designed to show whether M. dubius accumulates these amino acids and sucrose as distinct from determining the kinetics of the transport process. i.e. The K_m or K_t and V_{max} of the transport process. The amino acids tested were C^{14} labelled L isomers of leucine, alanine, serine and glutamic acid. C^{14} labelled sucrose, Chlorella protein and Chlorella protein hydrolysate were tested in the same way. The specific activity of the C^{14} labelled compounds used were leucine, 6.6 $\mu c/\mu M$, alanine, 20 $\mu c/\mu M$, serine 7.4 $\mu c/\mu M$, glutamic acid 130 $\mu c/\mu M$, Chlorella protein 200 $\mu c/\mu M$ and Chlorella protein hydrolysate 200 $\mu c/\mu M$. 0.25 microcurie of each compound was added to each culture medium. The Chlorella protein hydrolysate, supplied by the

Radiochemical Centre, Amersham, has the following composition.

Amino Acid	Per cent content
L - alanine	5
L - arginine	6
L - aspartic acid	6
L - glutamic acid	7
L - glycine	3
L - histidine	2
L - leucine	9
L - isoleucine	4
L - lysine	6
L - phenylalanine	4
L - proline	5
L - serine	2
L - threonine	2
L - tyrosine	4
L - valine	4
Nucleic acid	8
Total	77%

The experimental worms, which were all females, were equilibrated in Hanks balanced saline for 20 minutes at 37°C, and then transferred to 6.5 mls of labelled Hanks balanced salt solution, incubated separately for 2 hours, washed in Hanks balanced saline, dried and weighed. The control worms were killed by dropping them in boiling water for 15 seconds before incubation. After incubation

the worms were dissolved in 3 mls of a solution containing 50 : 1 of 2% $Na_2 CO_3$ in 0.1 N - NaOH and 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate. This solution was used to dissolve protein materials for nitrogen analysis by the Folin-Ciocalteu method. Twelve hours at room temperature proved sufficient to totally dissolve the worms. A 50 μl aliquot of this solution was dried on a flat aluminium planchet and counted using a Geiger-Muller tube and Echo N610A Scaler without pulse height analysis. Fifty μl aliquots of each separate culture medium were also counted to determine the concentration of C^{14} labelled amino acid in the medium . The method of calculation is shown in Appendix 6.1.

The accumulation of amino acids was estimated in terms of μM per gram dry weight of worm, per gram of worm water and per gram of nitrogen. Nitrogen content was estimated by the Folin-Ciocalteu technique and calculated as a percentage of the dry weight of worm, but significant differences were found between the nitrogen content of the body wall of various worms and for this reason it has not been used as a base for comparing the uptake of amino acids. Amino acid assimilation per gram of water in the worm has been used to present the results because it allows the calculation of a concentration factor, permitting comparison between worms and eliminating differences in worm uptake arising from differences between the concentrations of the media.

6.2.2. Rothman and Fisher (1964) estimated the Michaelis-Menten

parameters K_t and V_{max} for the accumulation of L - methionine, L - leucine, L - isoleucine, L - serine and L - alanine by male and female M. dubius. This work has been repeated using L - leucine as a test or substrate amino acid. Groups of worms were incubated in 12 different concentrations of L - leucine in Hanks balanced salt solution. The concentrations used were 10.0, 3.902, 1.951, 0.975, 0.878, 0.682, 0.585, 0.487, 0.390, 0.292, 0.195 and 0.098 mM or μM/ml. The worms were equilibrated in Hanks balanced salt solution for 20 minutes at 37°C before the test incubation was commenced. They were removed from the equilibration medium, dried, weighed and incubated in separate test culture media for 1 minute. Upon removal from the labelled media, all worms were rinsed in 3 x 500 ml, changes of Hanks balanced saline, dried and weighed. All worms were extracted for 24 hours in each of 3 changes of 2 ml of 95% ethanol.

Fifteen mls of dioxan : naphthalene : PPO cocktail were added to the samples and the counts taken with the Beckman CPM 200 liquid scintillation counter. The background count of ethanol extracts of worms taken from the rat gut, the ethanol itself, and the Hanks balanced salt solution was determined. Each of the media was counted to determine the ratio of C^{14} - U - L - leucine to C^{12} - L - leucine. This ratio was used to determine the total amount of L - leucine transported.

The methods of calculation are shown in appendices 6.2 and 6.3.

Two hundred μl aliquots of the ethanol extract were

analysed by 2 phase chromatography, using phenol saturated water (76% phenol) in the first phase and propionic acid/butanol/water in the second phase (46% propionic acid : 24% butanol : 30% water).

6.2.3.1. Read, Rothman and Simmons (1963) and Rothman and Fisher (1964) showed that active accumulation of amino acids by H. diminuta and M. dubius respectively, could be competitively inhibited by the presence of two or more amino acids if they share a common transport locus. The work of Rothman and Fisher has been repeated by estimating the uptake of C^{14} - U - L - leucine from 4 concentrations of this amino acid in Hanks balanced salt solution with and without 1.0 mM L - methionine.

The 4 test concentrations of C^{14} - U - L - leucine which were used contained 0.4, 1.0, 2.0 and 4.0 mM L - leucine and 1.0 mM L - methionine. Each culture contained 0.25 μc of C^{14} - U - L - leucine which had a specific activity of 6.6 μc/μM in Hanks balanced salt solution adjusted to pH 7.2. An atmosphere of air was used because the culture could not be satisfactorily gassed during the 1 minute incubation. For each level of C^{14} - U - L - leucine 6 cultures of 10 ml Hanks balanced salt solution were used, 3 for females and 3 for males. As a control for each concentration one worm of each sex was incubated in C^{14} - U - L - leucine alone. As a further control a small piece of sponge was incubated for 1 minute in individual cultures of the 0.4 and 4.0 mM L - leucine solutions.

The incubation of a piece of dry sponge in two of the test

cultures provides an estimate of the efficiency of recovery of the amino acid from the extraction and counting procedures. The sponge was weighed dry. It was incubated for 1 minute, placed in a tared screw cap counting bottle and reweighed. 2.0 mls of 95% ethanol was added and the C^{14} - U - L - leucine extracted for 24 hours. The extraction was replicated twice. The 3 extracts were counted and the amount of C^{14} - U - L - leucine determined. The weight of water absorbed by the sponge was known from the difference between the wet and dry weights. An estimate was made of the concentration of C^{14} - U - L - leucine in the water absorbed by the sponge. This was divided by the concentration of C^{14} - U - L - leucine in the incubation medium and the result expressed as a percentage. The results were:-

$$\frac{0.3940\ \mu M/ml}{0.3999\ \mu M/ml} \times \frac{100}{1} = 98.5\%$$

and

$$\frac{4.0204\ \mu M/ml}{4.000\ \mu M/ml} \times \frac{100}{1} = 100.5\%$$

The average recovery was 99.5%. Thus it has not been necessary to correct for recovery efficiency.

Other experimental details were the same as those given in section 6.2.2. The statistical methods used are shown in appendix 6.4.

6.2.3.2. A second competitive experiment was performed combining C^{14} - U - L - leucine with 16 other amino acids in Hanks balanced

salt solution. The amino acids were present in the molar ratios determined by Nasset and Ju (1961). They published 2 sets of amino acid ratios from rat gut samples taken 4 hours after feeding casein. These results have been averaged for each amino acid. These workers presented their results as ratios, using threonine (=1) as the base. The ratios have been recalculated to a base of 1 for leucine. A stock solution of amino acids in Hanks balanced salt solution was prepared which contained 4.0 mM L - leucine and appropriate amounts of the other amino acids to maintain the molar ratios. The method of preparation of the stock solution is shown in table 6.1. This stock solution was then diluted with Hanks balanced salt solution to give incubation media with L - leucine concentrations of 0.4, 1.0, 2.0 and 4.0 mM.

Six x 10 ml incubations were prepared for each leucine concentration and three worms of each sex were incubated individually. Six x 10 ml control media were prepared for each L - leucine concentration i.e. these media contained only leucine. For all test and control media three worms of each sex were incubated individually for each L - leucine concentration.

Other experimental methods and methods of calculation were the same as those given in section 6.2.3, but the recovery efficiency was not estimated.

TABLE 6.1. The method of preparation of the amino acid stock solution used for a competitive uptake experiment of L - leucine by M. dubius.

Amino Acid	Mean Molar Ratios	Adjust Ratio to Leucine = 1	Adjust Ratio to Leucine = 4 (A)	Mol Wgt (B)	Estimate as g/l ($AxBx10^{-3}$)
Alanine	3.18	1.177	4.708	89	0.4190
Arginine	2.09	0.774	3.096	174	0.5387
Aspartic Acid	1.13	0.419	1.676	133	0.2229
Glutamic Acid	3.28	1.215	4.860	147	0.7144
Glycine	2.14	0.792	3.168	75	0.2376
Histidine	0.17	0.063	0.252	155	0.0391
Isoleucine	1.11	0.411	1.644	131	0.2154
Leucine	2.70	1.000	4.000	131	0.5240
Lysine	1.85	0.686	2.744	146	0.4006
Methionine	0.59	0.219	0.876	149	0.1305
Phenylalanine	1.27	0.470	1.880	165	0.3102
Proline	1.07	0.396	1.584	115	0.1812
Serine	2.51	0.930	3.720	105	0.3906
Threonine	1.00	0.370	1.480	119	0.1761
Tyrosine	1.33	0.493	1.972	181	0.3569
Valine	1.17	0.433	1.732	117	0.2026

6.3. Results.

The results of this section are all given in tables. In the tables the column headed Diff. shows the difference between the means of the control and test groups. A positive difference indicates that the test group mean is greater than the control group mean and vice versa. The column headed L.S.D. (least significant difference) gives the tabulated "t" value, at the 5% level of probability with the appropriate number of degrees of freedom.

6.3.1. The first experiment, the results of which are in table 6.2, show the accumulation of 4 amino acids, sucrose, Chlorella protein and Chlorella protein hydrolysate from the culture medium.

The concentration factor given in the table is an underestimate of the accumulation because the worms have removed some of the nutrient from the culture medium, which reduces its concentration and overestimates the concentration gradient. Since the rate at which the nutrients assimilated are metabolised is unknown, the concentration of unaltered compound is also unknown. The concentration factors of the nutrients decrease with increasing molecular weight for dead worms, (table 6.3 and figure 6.1). The reverse is true for live worms with respect to the neutral amino acids, but the rate of change with molecular weight is greater for live than for dead worms.

TABLE 6.2. The concentration by female *M. dubius* of 7 C^{14} labelled nutrients from *in vitro* culture media after 2 hours incubation.

Nutrient and worm No.	Final worm nutrient content (μM/ml worm water)	Initial culture nutrient content (μM/ml medium)	Conc.** Factor	Average Conc. Factor (Live worms)
Leucine				
1	0.1458	0.005107	28.54)	
2	0.1290	0.005338	24.17)	26.13
3	0.1162	0.004523	25.69)	
*C	0.0098	0.005645	1.74	
Alanine				
1	0.01383	0.001263	10.95)	
2	0.01468	0.000911	16.12)	14.17
3	0.01223	0.000792	15.44)	
*C	0.00339	0.001735	1.95	
Serine				
1	0.03819	0.002446	15.61)	
2	0.03632	0.002431	14.94)	17.71
3	0.09584	0.004246	22.57)	
*C	0.01071	0.005991	1.78	
Glutamic Acid				
1	0.000348	0.000208	1.67)	
2	0.000830	0.000322	2.58)	2.19
3	0.000728	0.000315	2.31)	
*C	0.000531	0.000372	1.43	
Sucrose				
1	0.000514	0.003354	0.15)	
2	0.000956	0.003031	0.32)	0.21
3	0.000536	0.003369	0.16)	
*C	0.004505	0.003349	1.35	
Chlorella Protein				
1	0.01178	0.006485	1.82)	1.82
*C	0.00459	0.006656	0.69	
Chlorella Protein Hydrolysate				
1	0.24413	0.01462	16.70)	16.70
*C	0.02789	0.01706	1.63	

*C - is the dead worm, killed by boiling before incubation

** Concentration factor is calculated from the expression

$$\frac{\text{final concentration of nutrient in the worm } (\mu M/\text{ml worm water})}{\text{initial medium nutrient concentration } (\mu M/\text{ml of medium})}$$

TABLE 6.3. The ranking of compounds in decreasing order of mean concentration factor for live and dead M. dubius.

LIVE WORMS			DEAD WORMS		
Compound	Mean Conc. factor	Molecular Weight	Compound	Mean Conc. Factor	Molecular Weight
Leucine	26.13	131	Alanine	1.95	89
Serine	17.71	105	Serine	1.78	105
Alanine	14.17	89	Leucine	1.74	131
Glutamic Acid	2.19	152	Glutamic Acid	1.43	152
Sucrose	0.21	342	Sucrose	1.35	342

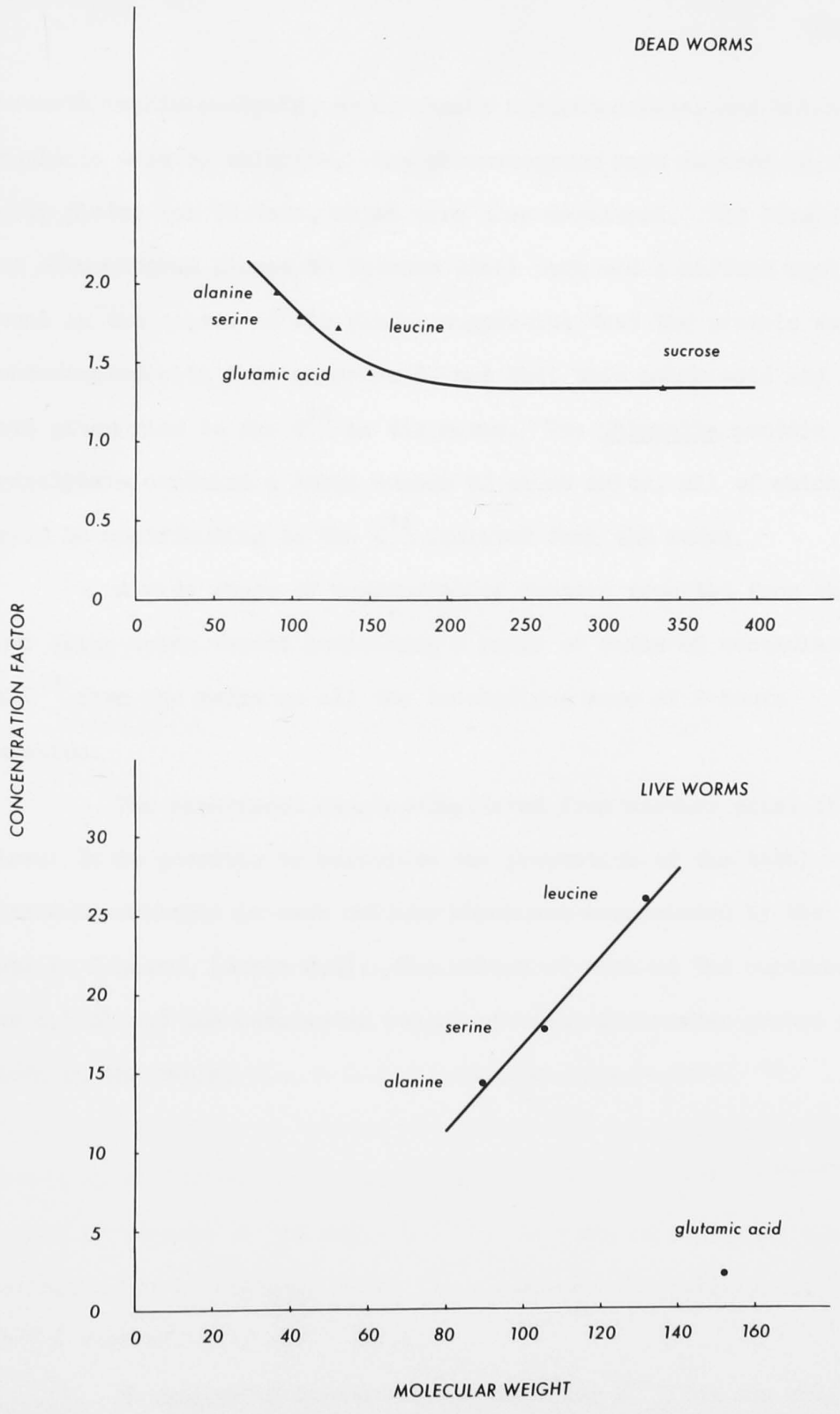
Since all the concentration factors for dead worms exceeded one it seems possible that the compounds were exchanging with their unlabelled counterparts and that equilibrium by diffusion had not been reached. It is unlikely that the correlation between molecular weight and the factor is coincidental.

The results with live worms show that the tegument of M. dubius is almost completely impermeable to sucrose, confirming the findings reported in chapter 2. Figure 6.1 also shows the relationship between concentration factor for live worms and the molecular weight of the amino acid. It can be seen that the concentration factors of the three neutral α amino acids are directly related to their molecular weight.

The ability of M. dubius to assimilate Chlorella protein was unexpected. The protein was subjected to two phase

FIGURE 6.1.

The relationship between amino acid molecular weight and female worm concentration factor for live and dead M. dubius.


DEAD WORMS
alanine
serine
leucine
sucrose
glutamic acid
2.0
1.5
1.0
0.5
0
0
50
100
150
200
250
300
350
400
LIVE WORMS
leucine
serine
alanine
glutamic acid
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
160
CONCENTRATION FACTOR
MOLECULAR WEIGHT

chromatographic analysis, using phenol saturated water and butanol/ propionic acid as solvents. The chromatograms were exposed to X-ray plates for 30 days, which were then developed. The origin of the chromatogram showed an intense black spot and a diffuse spot was found in the centre of the plate, suggesting that the protein was contaminated with free amino acid, and that this amino acid may have given rise to the C^{14} in the worms. The Chlorella protein hydrolysate contains a large number of amino acids, all of which could be contributing to the C^{14} isolated from the worms.

A wide range of concentration factors resulted from the four amino acids tested indicating a range of rates of accumulation of C^{14} from the media as all the incubations were of 2 hours duration.

The experiment can be considered from another point of view. It is possible to calculate the proportion of the total nutrient available in each culture which was accumulated by the worm in 2 hours, (table 6.4). The volume of each of the cultures was 6.5 ml and the mean water weight of each of the worm groups is shown in the table.

TABLE 6.4. The proportion of the available nutrient assimilated by *M. dubius* after two hours *in vitro* incubation and the mean weight of water in the worm.

Compound	Proportion assimilated %	Mean water weight of worms (g)
Leucine	47.3	0.1179
Alanine	26.9	0.1201
Serine	24.9	0.0975
Glutamic Acid	4.1	0.1208
Sucrose	0.33	0.1073
Chlorella protein	2.6	0.0945
Chlorella protein hydrolysate	13.8	0.0537

It can be seen that the mean weight of water in the worms is small compared to the volume of the culture and 47% of the available leucine was absorbed. It is noteworthy that the worm has located and assimilated such a high proportion of the available amino acid. It indicates that the carrier in the membrane must be able to combine rapidly with its substrate upon contact. The ability of the worm to contact and assimilate the amino acid may depend on diffusion of the amino acid down a concentration gradient caused by removal of the amino acid by the worm in its immediate vicinity, but by whatever means it is achieved the carrier must have a high affinity for leucine.

M. dubius is capable of accumulating C^{14} from the uniformly

C^{14} labelled amino acids leucine, alanine, serine and glutamic acid. The worm's ability to concentrate C^{14} from these amino acids is in the same order as the maximum transport velocities determined by Rothman and Fisher (1964). M. dubius is also capable of concentrating C^{14} from C^{14} - U - L - glutamic acid to a small degree. C^{14} from C^{14} - U - Chlorella protein hydrolysate is accumulated readily while radioactivity is incorporated from Chlorella protein, probably from contaminatory amino acids. M. dubius is almost totally impermeable to sucrose over the time span of the experiment.

6.3.2. The uptake of L - leucine per minute was determined using C^{14} - U - L - leucine. The uptake per minute and the medium concentrations used, are shown in table 6.5.

TABLE 6.5. The mean L - leucine uptake (μM/ml worm water/min) by M. dubius ($\pm$ 1 standard deviation) from various in vitro culture media concentrations.

Initial Leucine Concentration in medium (μM/ml)	No. Worms	Female: Leucine uptake	Male: Leucine uptake
0.0975	3	0.0539 $\pm$ 0.029	0.3219 $\pm$ 0.177
0.1949	3	0.1505 $\pm$ 0.035	0.1233 $\pm$ 0.005
0.2919	3	0.1328 $\pm$ 0.035	0.1906 $\pm$ 0.039
0.3901	3	0.1318 $\pm$ 0.042	0.1766 $\pm$ 0.008
0.4870	3	0.2096 $\pm$ 0.043	0.4727 $\pm$ 0.271
0.5849	3	0.2630 $\pm$ 0.025	0.4876 $\pm$ 0.249
0.6821	3	0.2942 $\pm$ 0.090	0.4446 $\pm$ 0.103
0.8779	3	0.2895 $\pm$ 0.108	0.6355 $\pm$ 0.396
0.9750	3	0.2631 $\pm$ 0.027	0.3131 $\pm$ 0.009
1.9510	3	0.3918 $\pm$ 0.061	0.9094 $\pm$ 0.456
3.9019	3	0.6616 $\pm$ 0.068	0.5351 $\pm$ 0.089
10.0001	3	1.7589 $\pm$ 0.398	2.5817 $\pm$ 2.071

FIGURE 6.2.

The relationship between L - leucine concentration and the uptake of L - leucine by female M. dubius after one minute incubation in Hanks balanced salt solution containing various concentrations of L - leucine.

The means are plotted. The vertical lines indicate $\pm$ 1 standard deviation. The curve is drawn by inspection.

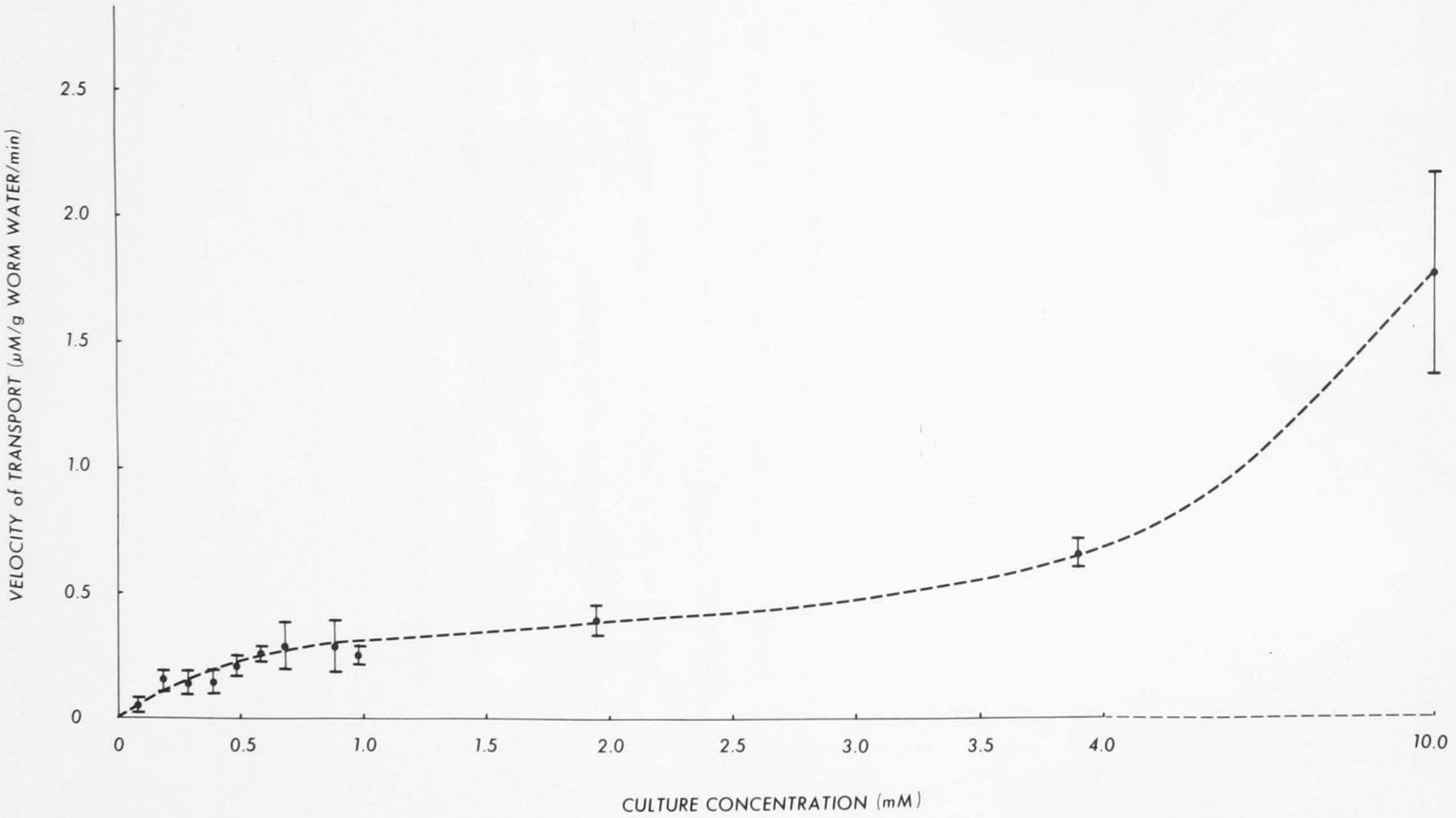
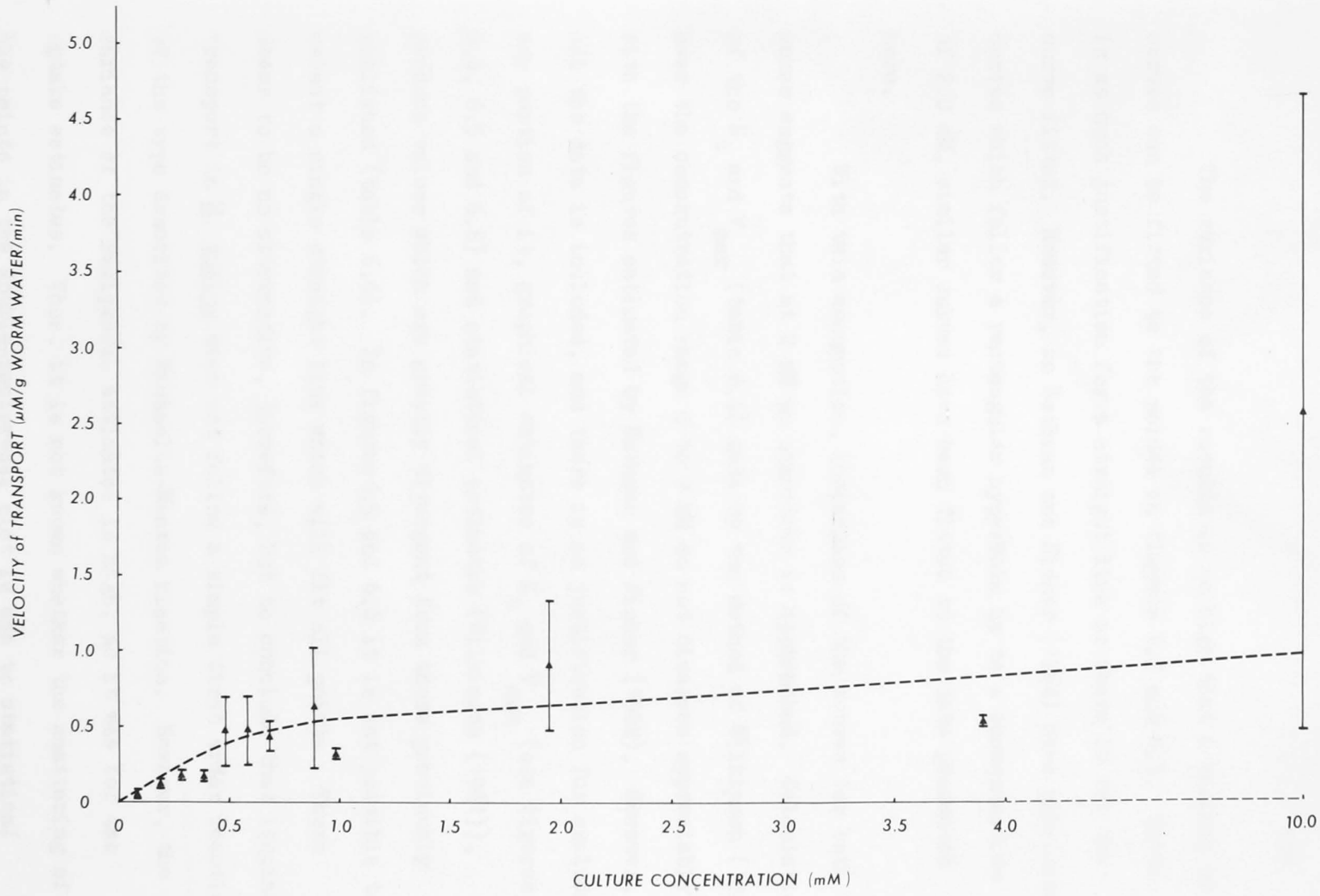

VELOCITY of TRANSPORT (μM/g WORM WATER/min)
2.5
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
10.0
CULTURE CONCENTRATION (mM)

FIGURE 6.3.

The relationship between L - leucine concentration and the uptake of L - leucine by male M. dubius after 1 minute incubation in Hanks balanced salt solution containing various concentrations of L - leucine.

The means are plotted. The vertical lines represent $\pm$ 1 standard deviation. The curve is drawn by inspection.

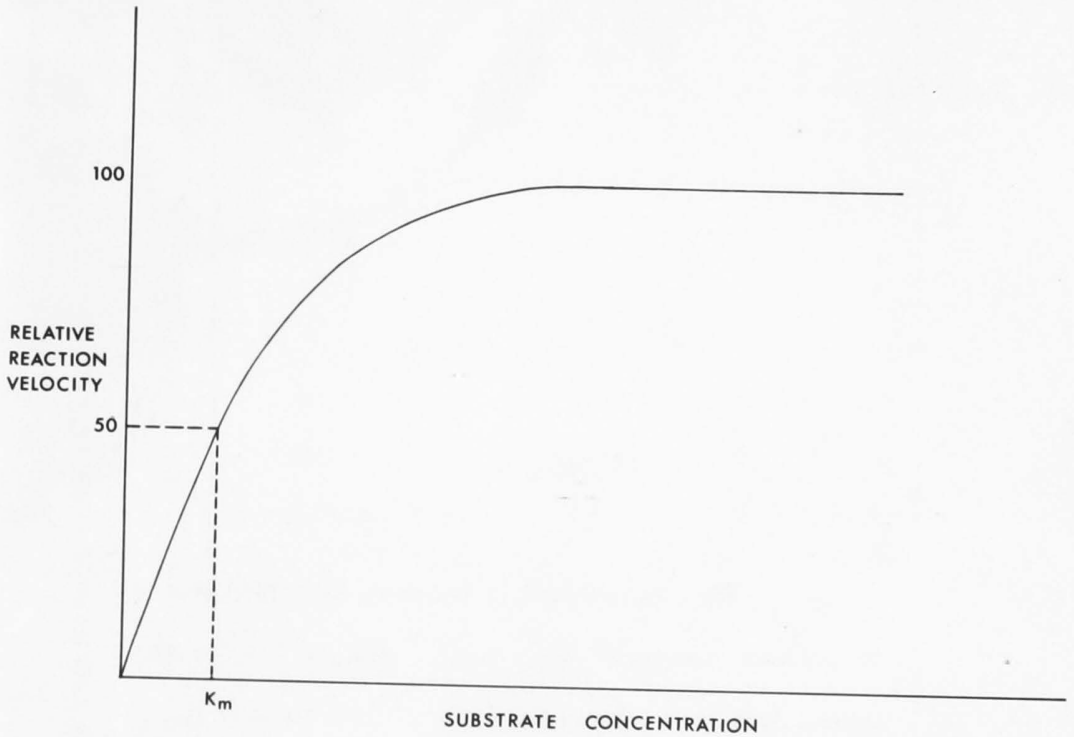

VELOCITY of TRANSPORT (μM/g WORM WATER/min)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
10.0
CULTURE CONCENTRATION (mM)

The variance of the results is so high that a variety of curves can be fitted to the points in figures 6.2 and 6.3. There is as much justification for a straight line as there is for the curve fitted. However, as Rothman and Fisher (1964) have published curves which follow a rectangular hyperbola up to a concentration of 2.0 mM, similar curves have been fitted to the data presented here.

With this assumption, inspection of the curves for both sexes suggests that at 2 mM an asymptote is approached. Calculations of the K_t and V_{max} (table 6.6) made by the method of Wilkinson (1961) over the concentration range 0 to 2 mM do not disagree appreciably with the figures estimated by Rothman and Fisher (1964). However, if all the data is included, and there is no justification for excluding any portion of it, graphical estimates of K_t and V_{max} (see figures 6.4, 6.5 and 6.6) and statistical estimates (Wilkinson (1961)), produce values which are greatly divergent from those previously published (table 6.6). In figures 6.5 and 6.6 it is not possible to select a single straight line which will fit all points. There seems to be no alternative, therefore, but to conclude that leucine transport in M. dubius does not follow a simple first order reaction of the type described by Michaelis-Menten kinetics. However, the variance of the reciprocal estimates is high, as it was for the uptake estimates. Thus, it is not known whether the scattering of the points in the double-reciprocal plot is due to statistical insufficiency or to real non-linearity of the relationship.

FIGURE 6.4.

The ideal relationship between the rate of a first order enzymatic reaction and the substrate concentration. The lower diagram shows the relationship of the reciprocals of reaction rate and substrate concentration for a series of points lying on the upper curve.


100
RELATIVE
REACTION
VELOCITY
50
Km
SUBSTRATE CONCENTRATION

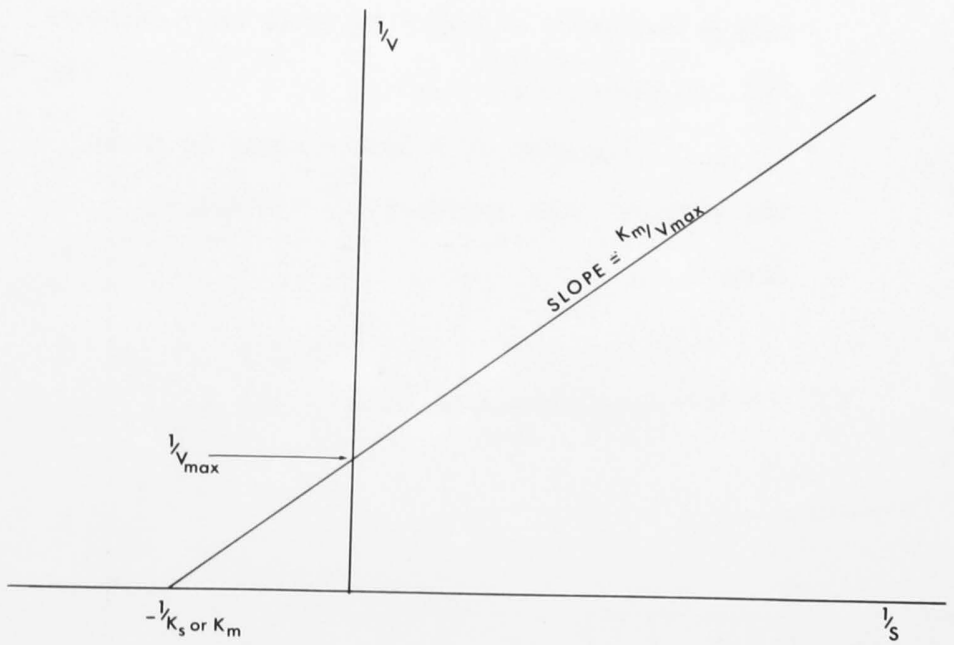

1/V
SLOPE = Km/Vmax
1/Vmax
-1/Ks or Km
1/S

FIGURE 6.5.

The relationship between the reciprocals of culture concentration $(mM)^{-1}$ and of L - leucine uptake $(\mu M/g$ worm water/minute$)^{-1}$ by female M. dubius incubated in Hanks balanced salt solution for 1 minute.

The mean of 3 female worms is shown. The vertical line represents $\pm$ 1 standard deviation.

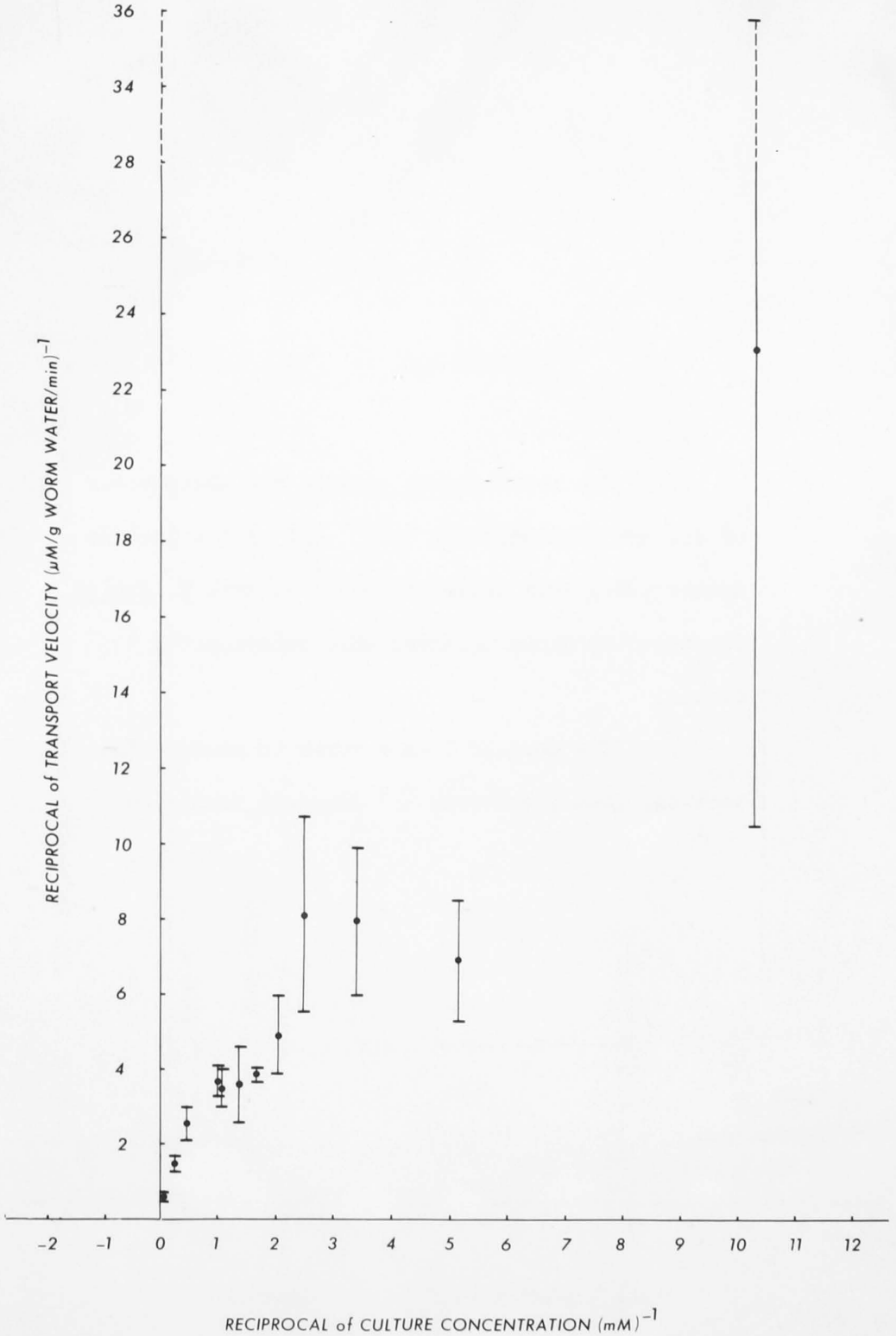

36
34
28
26
24
22
20
18
16
14
12
10
8
6
4
2
RECIPROCAL of TRANSPORT VELOCITY (μM/g WORM WATER/min)$^{-1}$
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
RECIPROCAL of CULTURE CONCENTRATION (mM)$^{-1}$

FIGURE 6.6.

The relationship between the reciprocals of culture concentration $(mM)^{-1}$ and of L - leucine uptake $(\mu M/g$ worm water/minute$)^{-1}$ by male M. dubius incubated in Hanks balanced salt solution for 1 minute.

The mean of 3 male worms is shown. The vertical line represents $\pm$ 1 standard deviation.

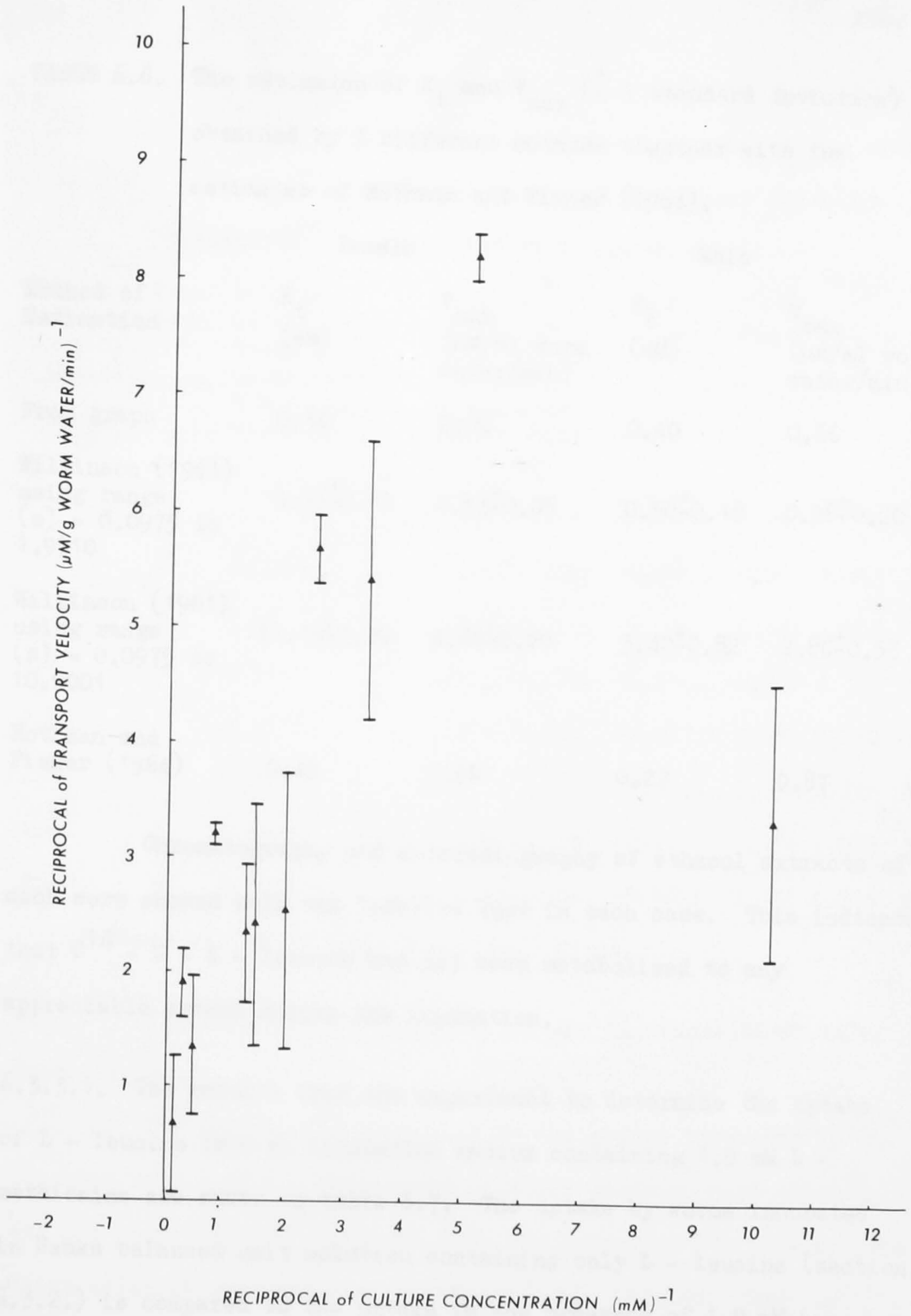

RECIPROCAL of TRANSPORT VELOCITY (μM/g WORM WATER/min)$^{-1}$
10
9
8
7
6
5
4
3
2
1
−2
−1
0
1
2
3
4
5
6
7
8
9
10
11
12
RECIPROCAL of CULTURE CONCENTRATION (mM)$^{-1}$

TABLE 6.6. The estimates of K_t and V_{max} ($\pm$ 1 standard deviation) obtained by 3 different methods together with the estimates of Rothman and Fisher (1964).

Method of Estimation	Female		Male	
	K_t (mM)	V_{max} (μM/ml worm water/min)	K_t (mM)	V_{max} (μM/ml worm water/min)
From graph	0.36	0.38	0.40	0.66
Wilkinson (1961) using range (s) = 0.0975 to 1.9510	0.71 ± 0.13	0.53 ± 0.05	0.58 ± 0.18	0.98 ± 0.20
Wilkinson (1961) using range (s) = 0.0975 to 10.0001	11.19 ± 1.70	4.80 ± 0.20	3.40 ± 0.82	2.80 ± 0.53
Rothman and Fisher (1964)	0.45	1.90	0.27	0.87

Chromatography and autoradiography of ethanol extracts of each worm showed only one labelled spot in each case. This indicates that C^{14} - U - L - leucine had not been metabolised to any appreciable extent during the incubation.

6.3.3.1. The results from the experiment to determine the uptake of L - leucine from an incubation medium containing 1.0 mM L - methionine are shown in table 6.7. The uptake by worms incubated in Hanks balanced salt solution containing only L - leucine (section 6.3.2.) is compared to the uptake in the presence of 1.0 mM L - methionine.

TABLE 6.7. The mean uptake of L - leucine from Hanks balanced salt solution (from section 6.3.2.), compared with the mean uptake ($\pm$ 1 standard deviation) from Hanks balanced salt solution containing 1.0 mM L - methionine.

Medium Leucine conc. (μM/ml)	Worm Sex	Leucine uptake from Hanks with no methionine (μM/ml worm water/min)	Leucine* uptake from Hanks with 1.0 mM methionine (μM/ml worm water/min)	Diff.	"t" Value	L S D	Sig.
0.4	F	0.1318	0.1934$\pm$0.090	+0.0616	1.077	2.776	NS
0.4	M	0.1766	0.1690$\pm$0.049	-0.0076	0.264	4.303	NS
1.0	F	0.2631	0.2540$\pm$0.029	-0.0091	0.399	2.776	NS
1.0	M	0.3131	0.3473$\pm$0.079	+0.0342	0.742	4.303	NS
2.0	F	0.3918	0.4692$\pm$0.147	+0.0774	0.085	2.776	NS
2.0	M	0.9094	0.9179$\pm$0.603	+0.0085	0.019	2.776	NS
4.0	F	0.6616	1.1573$\pm$0.778	+0.4957	1.103	4.303	NS
4.0	M	0.5351	0.7222$\pm$0.036	+0.1871	0.341	4.303	NS

* Means estimated from data from 3 worms.

The method of calculation for the "t" tests is shown in appendix 6.4.

The uptake of L - leucine by the groups incubated in the presence of 1.0 mM L - methionine in Hanks balanced salt solution was not significantly different from the uptake of L - leucine from Hanks balanced salt solution containing only leucine. Thus no evidence of competitive inhibition has been found. Only one

concentration of L - methionine (1.0 mM) was used. This was the amino acid concentration used by Rothman and Fisher (1964) in their experiments on competitive inhibition.

6.3.3.2. The results from an experiment to determine the uptake of L - leucine from Hanks balanced salt solution containing a complex mixture of amino acids are shown in table 6.8. The uptake by the worms incubated in Hanks balanced salt solution containing only L - leucine, was compared ("t" test) with the uptakes determined in section 6.3.2. No significant differences were detected so the uptake from complex culture has been compared with the uptake from the Hanks balanced salt solution media used as control groups in the experiment, the results are shown in table 6.8.

The results show that the presence of the other amino acids has not significantly altered the uptake of L - leucine. The total amino acid concentrations which were present for each of the L - leucine concentrations were : 3.9, 9.8, 18.7 and 39.4 mM i.e. the molar ratio of leucine to the other amino acids in the medium was kept constant.

TABLE 6.8. The mean uptake of L - leucine ($\pm$ 1 standard deviation) from Hanks balanced salt solution compared with the mean uptake of L - leucine ($\pm$ 1 standard deviation) from a complex amino acid medium in Hanks balanced salt solution.

Medium Leucine conc. (mM)	Worm Sex	Leucine uptake from Hanks with no other amino acids (μM/ml worm water/min)	Leucine * uptake from complex amino acid/Hanks culture (μM/ml worm water/min)	Diff.	"t" Value	LSD	Sig.
0.4	F	0.2260$\pm$0.057	0.1990$\pm$0.096	-0.0270	0.421	2.776	NS
0.4	M	0.3202$\pm$0.102	0.4227$\pm$0.572	+0.1025	0.304	4.303	NS
1.0	F	0.3149$\pm$0.018	0.4619$\pm$0.383	+0.1470	0.665	4.303	NS
1.0	M	0.5887$\pm$0.241	1.2644$\pm$1.944	+0.6757	0.598	4.303	NS
2.0	F	0.3932$\pm$0.033	2.6843$\pm$2.262	+2.2911	1.754	4.303	NS
2.0	M	0.7913$\pm$0.240	1.2474$\pm$1.059	+0.4561	0.727	4.303	NS
4.0	F	1.6610$\pm$1.621	0.6667$\pm$0.884	-0.9943	0.931	2.776	NS
4.0	M	0.6796$\pm$0.470	0.6755$\pm$0.834	-0.0041	0.009	2.776	NS

* Means estimated from data from 3 worms.

6.4. Discussion.

Figure 6.1 shows that the uptake of amino acids by dead worms is inversely proportional to the molecular weight of the amino acid, but this relationship does not apply to sucrose. The direct relationship between molecular weight and amino acid uptake by live worms may be due to the increasing length of the carbon

chain resulting in greater solubility of the amino acid : carrier complex in the lipid layer of the membrane. Alanine, which is least concentrated by the worm, has a 2 carbon chain, (CH_3 $CH(NH_2)$ COOH), serine which is concentrated to a greater extent than alanine has a 2 carbon chain with an hydroxyl group (HO. CH_2. $CH(NH_2)$. COOH) and leucine which is concentrated most has a 5 carbon chain ((CH_3)$_2$. CH. CH_2. $CH(NH_2)$.COOH). Glutamic acid does not fit this relationship, perhaps because it is a dicarboxylic amino acid (HOOC. CH_2. CH_2. $CH(NH_2)$ COOH). It would be interesting to see whether or not a similar relationship could be obtained between glutamic and aspartic acids. Danielli (1954b) discussed the nature of membrane bound carrier : substrate complex and pointed out that the complex must be hydrophobic for rapid translocation through the membrane. A hydrophobic complex would enter the lipoid moiety of the membrane more readily than a hydrophilic complex, but would experience greater difficulty in escaping into the aqueous interior of the cell or organism. The presence of serine with its terminal hydroxyl group in an intermediate position to alanine and leucine may be the result of this interaction.

The estimates of K_t obtained by the method of Wilkinson (1961), and using L - leucine concentrations up to 2.0 mM (section 6.3.2) are in reasonable agreement with those of Rothman and Fisher (1964). These authors determined K_t and V_{max} for the uptake of L - methionine, L - isoleucine, L - serine and L - alanine by male and female M. dubius. They used 3 minute incubations and detected up to

26% degradation of the amino acids. These authors do not give the concentrations of amino acids used in their culture media but they published a figure showing double reciprocal plots for the uptake of L - methionine by M. dubius with and without competing amino acids. The 1/S axis is calibrated from 0 to 15. The corresponding substrate concentrations from the calibrations 0.5, 5, 10 and 15 are $S = {}^{1}/_{0.5} = 2$ mM, $S = {}^{1}/_{5} = 0.2$ mM, $S = {}^{1}/_{10} = 0.1$ mM and $S = {}^{1}/_{15} = 0.07$ mM.

Thus it would appear that the highest substrate concentration used for methionine was 2 mM. Whether or not similar maximum concentrations were used for the other amino acids, including leucine, is not stated. However if V_{max} is estimated using a maximum substrate concentration of 2.0 mM reasonable agreement is obtained. Read, Rothman and Simmons (1963) determined similar parameters for the uptake of a range of amino acids by H. diminuta. The maximum amino acid concentration used in their kinetic studies was 1.0 mM. Thus all these workers used an experimental design which gave v against S curves similar to that shown in figure 6.4, under which conditions Michaelis-Menten kinetics may be applied. However, the amino acid concentrations used in the experiment, reported in section 6.3.2, show that the rate of uptake never became asymptotic, as far as can be determined from the data available.

Kilejian (1966) estimated that a large proportion of the uptake of L - proline by H. diminuta was by diffusion. She obtained

a non-linear relationship for the rate of uptake of L - proline by H. diminuta as the substrate concentration increased. She attributed this to the importance of diffusional uptake at substrate concentrations above 2.0 mM, and estimated the permeability constant of H. diminuta to L - proline by the method of Akedo and Christensen (1962). These workers derived the expression:

$$\frac{dAc}{dt} = Y + K_D (A_f - A_c)$$

where Y is the velocity of uptake by the carrier, which will be constant at high substrate concentrations, K_D is the apparent diffusion constant, A_f is the concentration of amino acid in the medium and A_c the concentration in the worm. Thus once the carrier is saturated the rate of uptake is a function of K_D and the concentration gradient, $(A_f - A_c)$. K_D may be estimated from the intercept of the straight line obtained by plotting $^1/A_f$ against the amino acid distribution ratio $A_c : A_f$. When the diffusional uptake was deducted from the total uptake a typical Micaelis-Menten reciprocal plot was obtained. An attempt was made to estimate the permeability of M. dubius to L - leucine so that a similar correction could be made. However, the rate of uptake at substrate concentrations up to 2.0 mM does not show a sharp discontinuity which would indicate that the active transport system was saturated. Thus it is impossible to determine the point at which diffusion became of appreciable importance. This made the estimation of a meaningful permeability constant impossible, so a correction for diffusion could not be made.

Rothman and Fisher (1964) estimated a series of inhibitor constants for the uptake of a series of amino acids in the presence of other amino acids. One of their determinations, competitive uptake of L - leucine in the presence of L - methionine, has been repeated in section 6.3.3.1. In section 6.3.3.2. the uptake of L - leucine from a medium containing a complex of amino acids was tested. Over the concentration range 0.- 4 mM L - leucine the uptake of L - leucine from Hanks balanced saline containing only this amino acid was not significantly different from the uptake from Hanks balanced saline containing a complex mixture of amino acids. The relationship between culture medium concentration and uptake was not suitable for the application of Michaelis-Menten kinetics so no attempt has been made to estimate an inhibitor constant. The data given in this chapter does not provide any evidence of competitive inhibition of leucine uptake, since the uptake is not significantly altered from that of the control worms by any of the conditions used.

The uptake data in table 6.5 shows that L - leucine uptake increased as the culture concentration increased. The absence of competitive inhibition could be due to the importance of diffusional uptake of amino acids, as there would be no inhibition between difusing amino acids. Alternatively, no evidence of competitive inhibition would be expected until all the transport sites were active. The media in the experiment performed with a complex mixture of amino acids contained far greater concentrations of

amino acid than the media containing only leucine. In the medium containing 4.0 mM L - leucine the total amino acid concentration was 39.4 mM. The other media were dilutions of this medium and had concentrations of 2.0, 1.0 and 0.4 mM L - leucine. The corresponding total amino acid concentrations were 18.7, 9.8 and 3.9 mM. The male worms showed an apparent uptake plateau at 2.0 mM L - leucine, or 18.7 mM total amino acid but the females do not show a detectable plateau up to 39.4 mM. This may be due to the importance of diffusional uptake, which would be a linear function of the concentration gradient. The smaller male worms may reach equilibrium with the media in 1 minute and thus show a plateau in the rate of L - leucine uptake, because further accumulation would only occur by active transport.

CHAPTER 7

THE ASSIMILATION OF L - LEUCINE BY M. DUBIUS (ACANTHOCEPHALA) FROM VARIOUS MEDIA.

7.1. Introduction.

Read, Rothman and Simmons (1963) tested the effect of modified external Na concentrations and Strophanthin G on the accumulation of amino acids by H. diminuta, as the involvement of Na in the transport of sugars (Crane, Forstner and Eichholz (1965)) and amino acids in some tissues had been suggested (Kuchler (1967)). They found that both Na level and Strophanthin G had no effect on the transport of L - methionine by H. diminuta.

It was known that M. dubius possessed mechanisms which allowed it to accumulate both Na and K ion when placed in NaCl and KCl solutions. Experiments were conducted to detect any involvement of the cation pumps in the accumulation of L - leucine.

7.2. Materials and Methods.

Two experiments were performed. The first employed 2 concentrations of K in a modified Hanks balanced saline in which the Na levels were adjusted to maintain the same real osmotic pressure. The second experiment compared the uptake of L - leucine from 300 mM sucrose compared with that from Hanks balanced saline at both 37°C and 4°C. Three male and 3 female worms were used in

each group in both experiments.

The modified Hanks balanced saline solutions used contained 111 mM Na and 28 mM K, referred to as Hanks + K, and 142 mM Na:0 mM K, referred to as Hanks - K and normal Hanks which (137 mM Na : 5 mM K) was used as a control medium. The experimental method and method of calculation was the same as shown in section 6.2.2 and appendices 6.2 and 6.4. Four L - leucine medium concentrations, 4.0, 2.0, 1.0 and 0.4 mM, were used.

In the second experiment the worms were equilibrated for 15 minutes in the sucrose solution from which the rate of uptake was to be determined. The equilibration time was reduced from the 20 minutes, used previously, to 15 minutes, to reduce the weight loss which occurs in sucrose because of the slow loss of cations by the worms.

The experiment was conducted at 37°C and 4°C, using 1.0 mM L - leucine in all the culture media. Apart from the change in equilibration time, the methods were the same as those given in section 6.2.2 and appendices 6.2 and 6.4.

7.3. Results.

7.3.1. The uptake of leucine from the 3 culture media used are shown in table 7.1.

The results also show a high coefficient of variation. The leucine uptake by worms in the Hanks control group did not

TABLE 7.1. The mean uptake of leucine (± 1 standard deviation) from 3 culture media and the comparison of the uptake from these media at 37°C.

Leucine culture conc. (mM)	Sex of Worm	Mean leucine uptake from Hanks balanced saline (μM/ml worm water/min) (1)	Mean leucine uptake from Hanks + K (μM/ml worm water/min) (2)	Diff.* (2-1)	"t" value	Sig.	Mean leucine uptake from Hanks - K (μM/ml worm water/min) (3)	Diff.* (3-1)	"t" value	Sig.
0.4	F	0.2260±0.057	0.1663±0.049	-0.0597	1.378	NS	0.1420±0.015	-0.0840	2.477	NS
0.4	M	0.3202±0.102	0.2898±0.029	-0.0304	0.497	NS	0.2279±0.041	-0.0923	1.452	NS
1.0	F	0.3149±0.018	0.2750±0.070	-0.0399	0.964	NS	0.2520±0.093	-0.0629	1.163	NS
1.0	M	0.5887±0.241	0.3896±0.090	-0.1991	1.350	NS	0.9418±0.576	+0.3531	0.986	NS
2.0	F	0.3932±0.033	1.0001±0.730	+0.6069	1.443	NS	0.4307±0.179	+0.0375	0.115	NS
2.0	M	0.7913±0.240	1.0199±0.647	+0.2286	0.579	NS	1.2328±1.485	+0.4415	0.509	NS
4.0	F	1.6610±1.621	0.6170±0.057	-1.0440	1.105	NS	0.5095±0.156	-1.1515	1.220	NS
4.0	M	0.6769±0.470	0.5921±0.235	-0.0848	0.291	NS	3.0471±3.801	+2.3702	1.076	NS

* A positive sign indicates that the test group uptake is greater than the control group uptake and vice versa.

differ significantly from that in Hanks balanced salt solution (section 6.3.2). The uptake for the two test groups has been compared to the control group in table 7.1. No significant change was detected in rate of uptake due to modification of the culture K concentration.

7.3.2. The second experiment compared the uptake of L - leucine from Hanks balanced saline and 300 mM sucrose at 37°C and 4°C. The mean rates of uptake are shown in table 7.2.

TABLE 7.2. The mean uptake of L - leucine by male and female M. dubius (± 1 standard deviation) from Hanks balanced saline (control) and 300 mM sucrose solution containing 1.0 mM L - leucine, at 37°C and 4°C.

Worm	Mean control group rate (μM/g worm water/min)		Mean test group rate (μM/g worm water/min)	
	37°C	4°C	37°C	4°C
Female	0.2466±0.020	0.0075±0.005	0.1465±0.029	0.0095±0.005
Male	0.3192±0.071	0.0397±0.046	0.1544±0.036	0.0870±0.035

The control group uptake at 37°C was not significantly different from the uptake established in section 6.3.2. To compare the uptake at 37°C from Hanks balanced saline and 300 mM sucrose, the uptake at 4°C, which represents the passive inflow, was deducted. The results of this adjustment are shown in table 7.3.

TABLE 7.3. Comparison of the mean uptake of L - leucine from Hanks balanced saline and 300 mM sucrose, adjusted for passive inflow at 4°C.

	Female	Male
Mean uptake from Hanks at 37°C *	0.2466	0.3192
Mean uptake from Hanks at 4°C *	0.0075	0.0397
Difference (A)	0.2391	0.2795
Mean uptake from sucrose at 37°C *	0.1465	0.1544
Mean uptake from sucrose at 4°C *	0.0095	0.0870
Difference (B)	0.1370	0.0674
Difference (C) = (A) - (B)	0.1021	0.2121
(C) as percentage of (A)	42.7%	75.9%

* uptake as μM/g worm water/min.

Thus the uptake by females is reduced by 43% in sucrose and, males by 76%. The corrected uptake for males and females in Hanks balanced saline was compared to the corrected uptake for males and females in sucrose. The results are shown in table 7.4. The above calculations were repeated to compare the uptake as μM/sq cm/min.

TABLE 7.4. Comparison of the corrected uptake of L - leucine by male and female M. dubius from Hanks balanced saline and 300 mM sucrose at 37°C.

Worm	Mean uptake from Hanks balanced saline (μM/g worm water/min) (1)	Mean uptake from sucrose (μM/g worm water/min) (2)	Diff. (1-2)	"t" value	Sig.
Female	0.2391	0.1370	-0.1020	7.19	*
Male	0.2795	0.0674	-0.2121	6.54	*
	(μM/sq cm/min) (1)	(μM sq cm/min) (2)	Diff. (1-2)	"t" value	Sig.
Female	0.01323	0.00627	-0.00696	23.9	**
Male	0.01145	0.00296	-0.00849	6.53	*

Thus the uptake from sucrose is significantly less than the uptake from Hanks balanced saline at 37°C.

The uptake at 37°C and 4°C from each medium was used to estimate the activation energy of L - leucine uptake by male and female M. dubius. The estimation of activation energy from a line drawn with only 2 points provides a provisional estimate only. The apparent activation energy was determined by the method of Bayliss (1959) by plotting the logarithm to the base e of the uptake against the reciprocal of the absolute temperature. The slope of the line is multiplied by the gas constant R (1.9871 cal/mole/°K) to give the apparent activation energy, E_A. The reciprocal of the absolute

temperature and logarithm to the base 10 of the uptake of L - leucine is shown in table 7.5 and the data presented graphically in figure 7.1.

TABLE 7.5. The reciprocal of the absolute temperature and $\log_{10}$ the uptake of L - leucine by male and female M. dubius.

Sex	Absolute Temperature	Reciprocal of absolute temperature	Hanks Balanced Saline: Leucine uptake	Hanks Balanced Saline: $\log_{10}$ Leucine uptake	300 mM Sucrose: Leucine uptake	300 mM Sucrose: $\log_{10}$ Leucine uptake
Female	310	0.00323	0.2466	-1.3920	0.1465	-1.1659
Female	277	0.00361	0.0075	-3.8751	0.0095	-3.9777
Male	310	0.00323	0.3192	-1.5041	0.1544	-1.1886
Male	277	0.00361	0.0397	-2.5988	0.0870	-2.9395

Thus the points to be plotted become:-

Reciprocal of absolute temperature	0.00323	0.00361
Leucine uptake from Hanks : female	-0.6080	-2.1249
" " " " : male	-0.4959	-1.4012
Leucine uptake from Sucrose : female	-0.8341	-2.0223
" " " " : male	-0.8114	-1.0605

The activation energy of L - leucine uptake from Hanks balanced salt solution and 300 mM sucrose was estimated from the rates of uptake expressed in terms of μM/g worm water/minute and μM/sq cm of body surface/minute. The results are shown in table 7.6.

FIGURE 7.1.

The relationship between the logarithm of the uptake of L - leucine (μM/g worm water/minute), by male and female M. dubius from Hanks balanced salt solution and 300 mM sucrose, and the reciprocal of the absolute culture temperature. (Females, full line; males broken line).

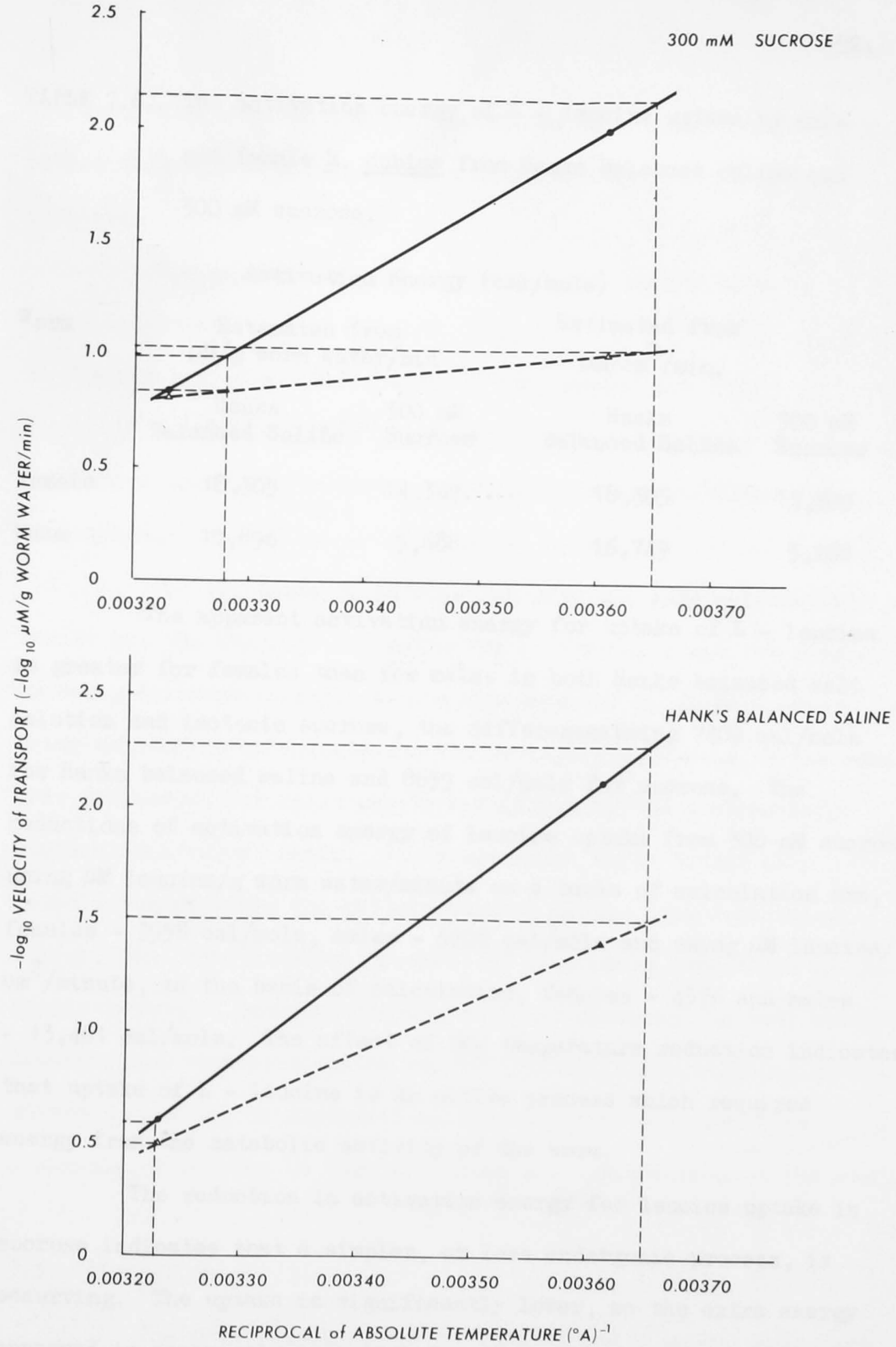

300 mM SUCROSE
HANK'S BALANCED SALINE
2.5
2.0
1.5
1.0
0.5
0
0.00320
0.00330
0.00340
0.00350
0.00360
0.00370
$-\log_{10}$ VELOCITY of TRANSPORT ($-\log_{10}$ μM/g WORM WATER/min)
RECIPROCAL of ABSOLUTE TEMPERATURE $(°A)^{-1}$

TABLE 7.6. The activation energy of L - leucine uptake by male and female M. dubius from Hanks balanced saline and 300 mM sucrose.

Worm	Activation Energy (cal/mole)			
	Estimated from μM/g worm water/min		Estimated from $\mu M/cm^2$/min.	
	Hanks Balanced Saline	300 mM Sucrose	Hanks Balanced Saline	300 mM Sucrose
Female	18,305	14,347	18,305	13,729
Male	10,896	5,688	16,749	3,268

The apparent activation energy for uptake of L - leucine is greater for females than for males in both Hanks balanced salt solution and isotonic sucrose, the differences being 7409 cal/mole for Hanks balanced saline and 8659 cal/mole for sucrose. The reductions of activation energy of leucine uptake from 300 mM sucrose using μM leucine/g worm water/minute as a basis of calculation are, females - 3958 cal/mole, males - 5208 cal/mole and using μM leucine/cm^2/minute, as the basis of calculation, females - 4576 and males - 13,481 cal/mole. The effect of the temperature reduction indicates that uptake of L - leucine is an active process which requires energy from the catabolic activity of the worm.

The reduction in activation energy for leucine uptake in sucrose indicates that a simpler, or less endergonic process, is occurring. The uptake is significantly lower, so the extra energy consumed is apparently essential for the rapid transport of L -

leucine. The lower activation energy for male worms may indicate either that a more efficient or a different type of process is involved.

The relationship of body weight to surface area is described by:-

surface area = $B.W.^{2/3}$ x 19.07 for females and

" " = $B.W.^{2/3}$ x 15.32 " males.

This shows that females have a greater surface area/volume ratio than males.

The two bases of calculation give the same comparative result but the absolute estimates are different except for females in Hanks balanced saline. As the estimate for males is greater using μM leucine/cm^2/minute and the results for females is the same, this difference for males may be a function of the smaller body surface area/volume ratio. It is not known which method of calculation provides the better estimate of the true activation energy. The concentration gradient of leucine is determined by the concentration of internal leucine which is dependent on body volume. However, the transport system may be located in the bounding membrane, so the uptake may be dependent on the number of carrier sites available, which may in turn depend on the surface area of the worm.

7.4. Discussion.

The involvement of the cations Na and K in the transport of glucose, (Crane, Miller and Bihler (1960), Crane, Forstner and

Eichholz (1965)) and amino acids (Kuchler (1967)) by various tissues, plus the knowledge that Na and K transport mechanisms existed in M. dubius led to the above experiments. The results show that the absence of K ion did not significantly affect the rate of accumulation of L - leucine by M. dubius. The significant reduction in the rate of uptake of leucine in sucrose suggested that at least one of the components of Hanks balanced saline is necessary for the active transport of L - leucine. The most likely component appears to be Na ion, but it has been shown by Ricklis and Quastel (1958) that the simultaneous presence of K and Mg ion in the lumen of guinea pig intestine was essential for the transport of glucose. It is hardly valid to compare glucose transport by the guinea pig intestine to L - leucine transport by M. dubius, but this finding demonstrates that Ca and Mg ions cannot be disregarded in such a discussion.

Read, Rothman and Simmons (1963) did not detect any effect of Na ion, or the cardiac glycoside Strophanthin G, on L - methionine transport by H. diminuta, suggesting that this cation is not involved in methionine transport by this cestode. This result also suggested that the amino acid transport mechanisms of these two animals may be basically different.

Rothman and Fisher (1964) did not test the influence of Na ion concentration on amino acid transport of M. dubius. They estimated Michaelis-Menten constants, i.e. K_t and V_{max}. The author has been unable to replicate these estimates satisfactorily,

as the rate of leucine uptake did not become maximal with increasing substrate concentration. Rothman and Fisher (1964) used a balanced salt solution containing 25 mM Tris (hydroxymethyl) aminomethane maleate buffer. Kolinska and Semenza (1967) discussed the possibility that "Tris" may compete with the Na ion for the allosteric site on the sugar carrier molecule, and that this competition would be competitive and would be difficult to distinguish from a fully competitive substrate inhibitor. Thus it is possible that Rothman and Fisher (1964), who used a "Tris" buffer, were examining either the kinetics of the competition between "Tris" and Na ion for the allosteric site on the carrier molecule rather than competition between the substrate amino acids, or a combination of both. Using 25 mM of buffer, and up to 2.0 mM of substrate, it is possible that the rate constant for amino acid uptake would be limited to the number of "Tris-free" sites. The number of free sites available would depend on the relative affinity of the allosteric site for "Tris" and Na ion. This is unknown. Rothman and Fisher (1964) determined inhibitor constants for several amino acids in "Tris" buffered media. Evidence has been presented in chapter 6 suggesting that the number of amino acid transport sites may increase as the concentration of amino acid available increases. Thus until a second plateau of amino acid uptake is reached there may not be any inhibition of rate of uptake. However competitive inhibition may be evident if the extra carrier sites were binding "Tris".

Read, Rothman and Simmons (1963) proposed 4 types of locus for amino acid uptake by H. diminuta, on the basis of the occurrence or non-occurrence of competitive inhibition between amino acids. This model may apply to M. dubius if it is assumed that the locus types are interchangeable to permit the rapid transport of a large amount of one amino acid.

Schafer and Jacquez (1967) demonstrated that the rate of uptake of one amino acid by Ehrlich ascites tumor cells was stimulated by the extracellular presence of another amino acid. This process of competitive stimulation resulted in an increased rate of transport for one of the amino acids, and a decreased rate for the other as compared to their rates when present alone.

Jacquez (1967) demonstrated the presence of 2 amino acid transport systems which function during competitive stimulation of amino acid uptake in Ehrlich ascites tumor cells. One system gives a rapid uptake of one amino acid and the second gives an exchange counterflow of the other amino acid. Inui and Christensen (1966) published strong evidence for a two carrier system, one of which was Na ion dependent, in ascites tumor cells. Oxender and Christensen (1963) described these as the A and L systems. The A system was a poorly exchanging system which serves for primary uptake of neutral amino acids. When 2 amino acids were present the rate of uptake by the A system was greatly increased and the L system carried the other amino acid by exchange diffusion. Inui and Christensen (1966) showed that the A system was dependent on the presence of Na ion.

Whether or not 2 amino acid carrier species exist in M. dubius is not known, but this type of transport complex may explain the increased rate of leucine uptake with increasing culture medium concentrations.

The existence of an amino acid transport mechanism in M. dubius which obeys Michaelis-Menten kinetics has not been substantiated, perhaps because of the high variance encountered in estimates of the rate of uptake, or perhaps because the number of transport loci increases with the concentration of substrate, or a two carrier system of some type is involved. Similar reasons may account for the absence of demonstrable amino acid competitive inhibition in amino acid transport. From the above discussion it is concluded that the entire matter of amino acid transport by M. dubius requires re-examination using "Tris" free media and higher amino acid concentrations.

The suggested involvement of Na ion in the transport of amino acids plus the presence of high concentrations of Na ion at the level of the basement membrane may suggest that a transport system exists on the basement membrane to transfer amino acids from the body wall to the pseudocoel.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS.

M. dubius is normally parasitic in the small intestine of the rat, an environment which, although extracellular, shows little variability. Nasset and Ju (1961) demonstrated that the relative ratios of amino acids remain constant in the rat gut regardless of the diet of the rat or its absorptive state. The author has determined that the pH of the gut contents, in the region of the worm, is stable at 7.2, and that the Na and K concentrations remain constant. As the rat is the normal definitive host of M. dubius it would be expected that the parasite is closely adapted to the environment provided by the rat gut. In addition the worm must be equipped with sufficiently adequate nutrient uptake mechanisms to allow it to compete effectively with the host for nutrients. This is supported by the finding of Burlinghame and Chandler (1941) that there is a zone of viability for M. dubius which extends from 10 to 60 cm below the pyloric sphincter. Should M. dubius find itself outside this zone it cannot survive, presumably because it cannot obtain the required nutrients. Thus, if this parasite is to survive it must be located at a point in the intestine where it is adequately supplied with nutrient materials and can effectively compete with the assimilatory mechanisms of the host.

M. dubius is a morphologically simple animal. It does not possess a gastrointestinal tract, an identifiable excretory system

nor a secreted, protective cuticle, as for example, do nematodes. In M. dubius only the cuticle has a structural analogue, the epicuticle. The complete loss of a digestive tract is a specific adaptation to parasitism. The loss of specialised osmoregulatory and excretory organs could also be considered to bea parasitic adaptation. The epicuticle of M. dubius is a mucopolysaccharide meshwork which is about 1 μ thick. The bounding living layer is a plasma membrane below which is a sub-plasma membrane. The sub-plasma membrane is penetrated by the pores of the canals of the striped layer. The canals are lined by the plasma membrane (Nicholas and Mercer (1965)). This structure suggests that the plasma membrane is the only continuous living layer between M. dubius and its environment, and indicates that the homeostatic mechanisms of M. dubius must be located in this membrane.

The plasma membrane of M. dubius is described by Nicholas and Mercer (1965) as ".... a typical plasma membrane ....". If it is typical in the functional sense as well as the structural sense, it would be expected to be semipermeable (Willmer (1961)). This proved to be the case (chapter 2). M. dubius behaves as an imperfect osmometer in hyperosmotic and hypo-osmotic sucrose solutions, indicating that the bounding membrane permits the passage of water, but not of solutes. Further evidence of the functionally typical nature of this membrane is furnished by the estimate of its permeability constant for water, obtained using H_2^3O (chapter 5). The permeability of the bounding membrane to water is similar to that of

the plasmalemma of soft body tissue cells of other animals. The permeability constant was determined by calculating the surface of the worm, assuming that it was cylindrical. If the canals of the striped layer are in fact lined by the bounding membrane, they may greatly increase the surface area of the worm. Thus the permeability constant determined may be an overestimate.

The semipermeable nature of plasma membranes is usually attributed to hydrated pores which penetrate the membrane. Conway (1954) considered that these pores give membranes their capacity to act as molecular sieves, permitting the penetration of small ions. Similar pores would be expected in the plasma membrane of M. dubius. Experiments showed that the plasma membrane was permeable to Cl, CO_3 and HCO_3 ions, slightly permeable to NO_3 ion and impermeable to SO_4 ion. The hydrated radii of these ions are, Cl - 2.4 Å, CO_3 - unknown, HCO_3 - 4.2 Å, NO_3 - 2.6 Å and SO_4 - 4.6 Å (Eccles (1965)). That the membrane is permeable to HCO_3 ion but impermeable to SO_4 ion suggests a pore radius of 4.2 to 4.6 Å. A pore diameter ranging from 8.4 to 9.2 Å is similar to the mean pore diameter, 8 Å for muscle membranes given by Conway (1954). The hydrated radius of Ca and Mg ions, are 9.6 Å and 10.8 Å (Potts and Parry (1964)). These ions do not readily penetrate the plasma membrane of M. dubius, which is consistent with the estimated pore diameter of 8.4 to 9.2 Å.

The membrane of M. dubius is however only slightly permeable to NO_3 ion with a hydrated radius of 2.6 Å. Conway (1954) described a similar situation in muscles. The Cs ion has a hydrated radius of

3.6 Å and K a hydrated radius of 3.8 Å. However K ion penetrates muscle cell membranes far more rapidly than the smaller Cs ion. Conway concluded that some factor other than hydrated ionic radius is involved in the ability of ions to penetrate membranes. Potts and Parry (1964) give a list of hydrated ionic radii. The hydrated radius of the NO_3 ion is given as 0.95 Å, (c.f. Eccles (1965) 2.6 Å); Cl as 1.9 Å (c.f. Eccles 2.4 Å) and SO_4 as 3.6 Å (c.f. Eccles 4.6 Å). Potts and Parry comment that the effective hydrated radius of an ion depends upon the degree of saturation, which can depend on even the hydrostatic pressure applied to the solution. The hydrated radius also depends on the method by which it is estimated. Thus, an estimate of 8 - 9 Å for the diameter of the pores in the bounding membrane of M. dubius may not be accurate because of the variable nature of the radii of hydrated ions.

The plasma membrane of M. dubius is therefore similar to other plasma membranes in this respect. It would then be expected that the membrane would permit the rapid exchange of water. The evidence obtained from work with H_2^3O indicated that this is so (chapter 5). It was determined that water moves rapidly through the membrane in both directions, so it would be expected that the temperature coefficient of water movement in both directions would be the same. It has been shown that the decrease in rate of movement with temperature in both directions is the same, indicating that the activities of water in the worm, and in the medium, are also similar. In other words there can be no osmotic gradient across

the membrane. This is confirmed by the observations that both Hanks balanced salt solution and the rat gut are isotonic with M. dubius because the worm remains flaccid in both environments. Tosteson (1963) described a similar situation in tissue cells when he pointed out that a tissue cell would rapidly shrink or rapidly swell if an osmotic gradient was applied across its bounding membrane.

It is therefore clear that M. dubius is in osmotic equilibrium with its environment. It was impossible, at first, to reconcile this observation with the result of Na, K, Ca and Mg analysis of the worm tissues (chapter 3). This analysis provided estimates of the osmotic pressures that these ions would exert if they were present as chlorides. This assumption was made because the presence of Cl ion accompanying Na and K was demonstrated by the histochemical investigations of ionic distribution. Calculations of osmotic pressure on this basis showed that whole males and the tegument of females were hyperosmotic to the rat gut and Hanks balanced salt solution. Experimental determinations of osmotic pressure, by measurement of the freezing point depression of tissues and pseudocoel fluids, also showed these components to be hyperosmotic. The apparent conflict of results can be resolved by making the assumption that the cations in whole males and in the body wall of females are distributed between two physiologically distinct compartments. One of these compartments contains osmotically active cations and the other osmotically inactive cations. The osmotically active component should also be iso-osmotic with the rat gut and

Hanks balanced saline.

Support for the hypothesis came from an investigation into the physiological behaviour of the cations in whole males and in the body wall of females, (chapter 4) which showed that there were, in fact, two pools of Na and K. One a readily exchangeable pool proved to be iso-osmotic with the rat gut and Hanks balanced salt solution. The second, a non-exchangeable pool, appeared to be osmotically inactive.

The pseudocoel of female M. dubius was found to be iso-osmotic with the rat gut and Hanks balanced salt solution with respect to total cations calculated on the basis that they were present as chlorides. The proportion of these cations which was exchangeable was not determined because the exchange had not reached equilibrium with the Hanks balanced saline over 4 hours. However, because the pseudocoel is iso-osmotic with the rat gut it may be predicted that all the cations of the pseudocoel fluids, but not necessarily of the eggs, would exchange, although more slowly than those of the body wall.

Glynn (1959) observed that if cell Na and K are bound a proportion of the cell water must also be bound to maintain osmotic equilibrium. It has been shown that a proportion of the cations of the female body wall and whole males are bound in M. dubius. In order to determine whether or not Glynn's observations were also valid for M. dubius, experiments with tritiated water were undertaken. A proportion of the water in the female body wall and in the whole

male proved to be non-exchangeable which indicates that there must, in fact, be two physiologically distinct pools of water. In addition female pseudocoel exchanges 93% of its water, which again suggests that the majority of the pseudocoel cations would exchange if a sufficient period was allowed, and agrees with the finding that the total pseudocoel cations produce isotonicity with the rat gut. The ability of the pseudocoel to exchange its cations slowly, while rapidly exchanging its water, indicates that the basement membrane is also a semi-permeable membrane and that the pseudocoel therefore behaves as a coelom. The environment provided for the eggs is thus of stable cation composition; it has also been shown that the pseudocoel rapidly accumulates amino acids thus providing both the stable environment and the continuous supply of nutrient materials which are necessary for the growth and development of the eggs. The adult female is virtually a sac in which fertilisation and embryological development proceeds, culminating in the production of acanthors enclosed in a resistant shell. Nicholas and Grigg (1965) showed that embryonic development is dependant on the controlled internal environment of the pseudocoel. This evidence confirms that these characteristics are of the utmost importance in the production of shelled acanthors.

The equilibration of the exchangeable water of M. dubius with Hanks balanced saline is rapid. A functional osmoregulatory system has not been identified in M. dubius so it would be expected that the exchange of water would occur via the plasma membrane which

mediates between the organism and its environment. The demonstration that water exchange takes place over the whole surface of the animal confirms that this is so. The only other route of exchange that is possible is via the reproductive system. Experiments show that the reproductive system is not involved and this can be rationalised with the morphological structure and function of the reproductive system of both sexes. The female reproductive system is open interiorly to the pseudocoel fluids, but exchange of water by this path could not occur while the copulation plug inserted by the male is in position. The reproductive system of the male would be unable to provide a route of exchange because the pseudocoel fluids do not have access to the canals of the system.

It has thus been shown that the exchangeable cations of M. dubius maintain the animal in an osmotic equilibrium with its environment. In tissue cells the Na and K ions exert a similar effect because they are distributed in a double-Donnan equilibrium which is maintained by Na and K exchangeable pumps. The concentrations of Na and K in M. dubius and the cations in the rat gut are distributed in the same way as they are between tissue fluids and cells of the rat. The gut which may be considered to be equivalent to the tissue fluids, contains a high concentration of Na and a low concentration of K, while the body wall of the worm, equivalent to the cells, maintains a high concentration of K and a low concentration of Na. This distribution of cations suggested that a Na and K pump system would be present in the tegument of M.

dubius.

If the osmotic control mechanism of M. dubius is similar to that of tissue cells, there would be a continuous exchange of Na and K between the animal and its environment as the passive movement of cations through the bounding membrane is compensated by the active movement of cations in the opposite direction. This has been shown to occur in Hanks balanced salt solution. A rapid exchange of water would be expected to accompany this exchange of cations because of the hydration shell of the ions. Such an exchange of water has been observed over the entire body surface. Any osmoregulatory system therefore, must be located in the vicinity of this membrane.

The canals of the striped layer could provide the necessary physical structures for an osmoregulatory system of the type suggested by Maddrell and Treherne (1967) and Berridge and Gupta (1967). These workers proposed a system for active movement of water in the perineurium of Carausius morosus and Periplaneta americana and in the rectal papillae of Calliphora sp. respectively. Essentially, the system consists of a canal, which is blind at the proximal end and open at the distal end. A Na or K transport pump establishes a high solute concentration in the blind end of the canal, resulting in an inflow of water. The resulting increase in hydrostatic pressure in the canal is relieved by drainage of water out of the open end of the canal. The cations are reabsorbed and recirculated by the transport pumps. Such a system therefore

involves the rapid re-accumulation of Na and K, and requires a readily available supply of Na and K at the level of the plasma membrane. The tegument of M. dubius can fulfil both of these conditions. The Na and K observed at the level of the basement membrane may similarly achieve circulation of water into and out of the pseudocoel. Crompton and Lee (1965) speculated on the folded nature of the basement membrane of Polymorphus minutus, an acanthocephalan parasitic in birds, and consider that it resembles those of tissues specialised for the transport of ions and water.

The plasma membrane of M. dubius has thus been shown to be responsible for the maintenance of water and salt content in the organism. Subsequent experiments showed that the same membrane is responsible for the accumulation of at least some nutrients, because M. dubius actively accumulates amino acids from in vitro media. The presence of an active mechanism for the accumulation of amino acids is not surprising when it is considered that the worm must compete with the host gut, and any other parasites in the gut, for the available nutrients. The finding that amino acids are also accumulated by the pseudocoel is to be expected as this component of the worm is engaged in the rapid production of acanthors, for which it must need structural materials.

Rothman and Fisher (1964), on the assumption that Michaelis-Menten kinetics applied to transport across the membrane, estimated K_t and V_{max} for the absorption of amino acids by M. dubius. Approximately the same estimates are reached by selecting data from

the experiments (chapter 6) to give the same amino acid substrate concentrations as those used by Rothman and Fisher. The data from these experiments indicated however, that first order kinetics are not adequate to describe this process. This may be due to the contribution of diffusional uptake of amino acids of the type suggested by Kilejian (1966). However it is difficult to understand how an amino acid would diffuse through a membrane with the semi-permeable characteristics of the bounding membrane of M. dubius. An alternative explanation of the continuing increase in the rate of uptake of amino acid with increasing medium concentration is that the number of amino acid carrier sites in the membrane of M. dubius may increase in response to the external amino acid concentration.

Nasset and Ju (1961) determined that the relative molar ratios of the contents of the rat gut remain stable. It would be expected however, that changes would occur in the concentration of amino acid in the gut as digestion of a meal continues. When this factor is considered together with the competition from the rat gut and from other worms, a system of amino acid carriers which does not readily become saturated with amino acids, such as that described in the preceding paragraphs, can be justified. When several female M. dubius are present side by side in the rat gut, a large proportion of the worms absorptive surface may be in contact with other worms or with the gut wall. It may therefore be advantageous for the parasite to be able to absorb nutrients rapidly through that part

of its surface which is in contact with the ingesta and accompanying fluids.

Two other factors must also be considered. The first is the significance of the canals of the striped layer. These canals are lined with plasma membrane and may provide an increased absorptive surface. If this is so, it would be necessary to exchange constantly the contents of these canals. This would be particularly true if the canals also serve the function of an osmoregulatory system. Exchange of the canal contents may be jointly achieved by the peristaltic waves of the gut and muscular movements of the worm. The peristaltic waves of the gut would have the effect of squeezing out the canal contents and then carrying the expelled contents along the surface of the worm. The gut contents behind the wave would then move downwards and provide fresh material for the canals to absorb as the body of the animal relaxes. Muscular movements by the worm would exert a similar effect. Under in vitro conditions the amount of muscular activity could thus influence the rate of nutrient assimilation.

Similar considerations may have caused the failure of the experiments designed to detect the competitive inhibition of amino acid uptake observed by Rothman and Fisher (1964). Other reasons for this difference in results may be the shorter incubation time used here, 1 minute against 3 minutes for Rothman and Fisher, the strain of worms used and the buffer system used.

The plasma membrane has been shown to be responsible for

the active exchange of cations and the active uptake of amino acids by M. dubius. Evidence has been presented suggesting that amino acid accumulation may be dependant on the Na and K transport systems. External K ion alone exerted no effect on amino acid accumulation, but the simultaneous absence of Na, K, Ca and Mg did significantly reduce the rate of uptake of L - leucine by M. dubius. It is interesting to note that the Na and K transport system operates continuously, and may provide a continuous coupled system for amino acid uptake. This feature would also be advantageous in competition with other worms and the host.

The above discussion shows that M. dubius has adapted itself to its host in the same way as the host's cells are adapted to its body fluids i.e. there appears to be complete harmony between host and parasite. Further M. dubius does not provoke any detectable immune response by the host and causes very little gut damage (Burlinghame and Chandler (1941)). Thus this animal is ideally adapted to its host despite its apparent morphological degeneracy. During the accumulation of data for this thesis a large number of incidental problems have arisen. Some of these deserve investigation, for example, the purely physical effects of the peristaltic waves of the gut and muscular movements by the worm. The significance of the epicuticle also deserves attention. This structure could have three functions. Firstly, it may simply enclose solid materials passing the worm and thus afford protection to the

membrane. Secondly, it may bear a fixed charge which electrostatically binds molecules until they are absorbed; thirdly, it may maintain a film of fluid, free of particles, over the surface of the worms which allows exchange to occur. The characteristics of the cation pumps could be further investigated with the use of micro-electrodes and the other specialised equipment and techniques of this field. The suggestion advanced by Nicholas and Mercer (1965) and Edmonds and Dixon (1966) that M. dubius may assimilate nutrients by pinocytosis, as well as the possibility that reversed pinocytosis is responsible for excretion, deserve further examination. These are but some of the questions raised by this thesis, which would form the bases of several separate research programmes.

It has however been shown that a harmonious and dynamic relationship exists between M. dubius and its rat host. Five conclusions may be drawn from the data. Firstly, M. dubius depends on its external plasma membrane to mediate the exchange of material between itself and its environment. Secondly, this membrane is a typical plasma membrane which is semipermeable and is equipped with active cation pumps. Thirdly, only the Na and K ions which are exchangeable are osmotically active and that they produce isotonicity with the environment. The remaining non-exchangeable cations are physiologically separate from the exchangeable cations. Fourthly, any osmoregulatory capacity of M. dubius depends on the cation pumps of the plasma membrane through which all water exchange occur.

Finally, M. dubius is capable of accumulating amino acids in both the body wall and pseudocoel.

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APPENDIX 3.1.

The method of calculating the weight of cation per gram of worm water in an analysed component is as follows:-

A standard optical density/concentration curve is constructed from the Na and K standard solutions. The concentration of cation in the diluted unknown solution may be determined by interpolation on the graph shown in appendix figure 3.1 for Na and figure 3.2 for K. A sample unknown value has been taken from each of the graphs. Pseudocoel Na gives a concentration of 1.15 ppm from an absorbance of 0.22, and body wall K gives a concentration of 18.2 ppm from an absorbance of 0.35. These solutions have a volume of 50 mls and 25 mls respectively, and the weight of water in the samples was 0.0472 gram and 0.0881 gram respectively.

If the interpolated value from the standard curve is y ppm Na or K, then there are y g Na or K per 10^6 g worm water. The volume of the solution is a mls, therefore in a mls there are

$$y \times a \times 10^{-6} \text{ g Na or K}$$

$\therefore$ this amount of Na or K was originally contained in n g of worm water in the tissue analysed.

$$\therefore \text{ g Na or K / g tissue water} = \frac{y \times a \times 10^{-6}}{n}$$

Applying the above examples; for pseudocoel Na:

$y = 1.15$ ppm.

$n = 0.0472$ g.

$a = 50$ mls.

$$\therefore \text{ g Na / g tissue water} = \frac{1.15 \times 50 \times 10^{-6}}{0.0472} = 0.00122 \text{ g Na/g}$$

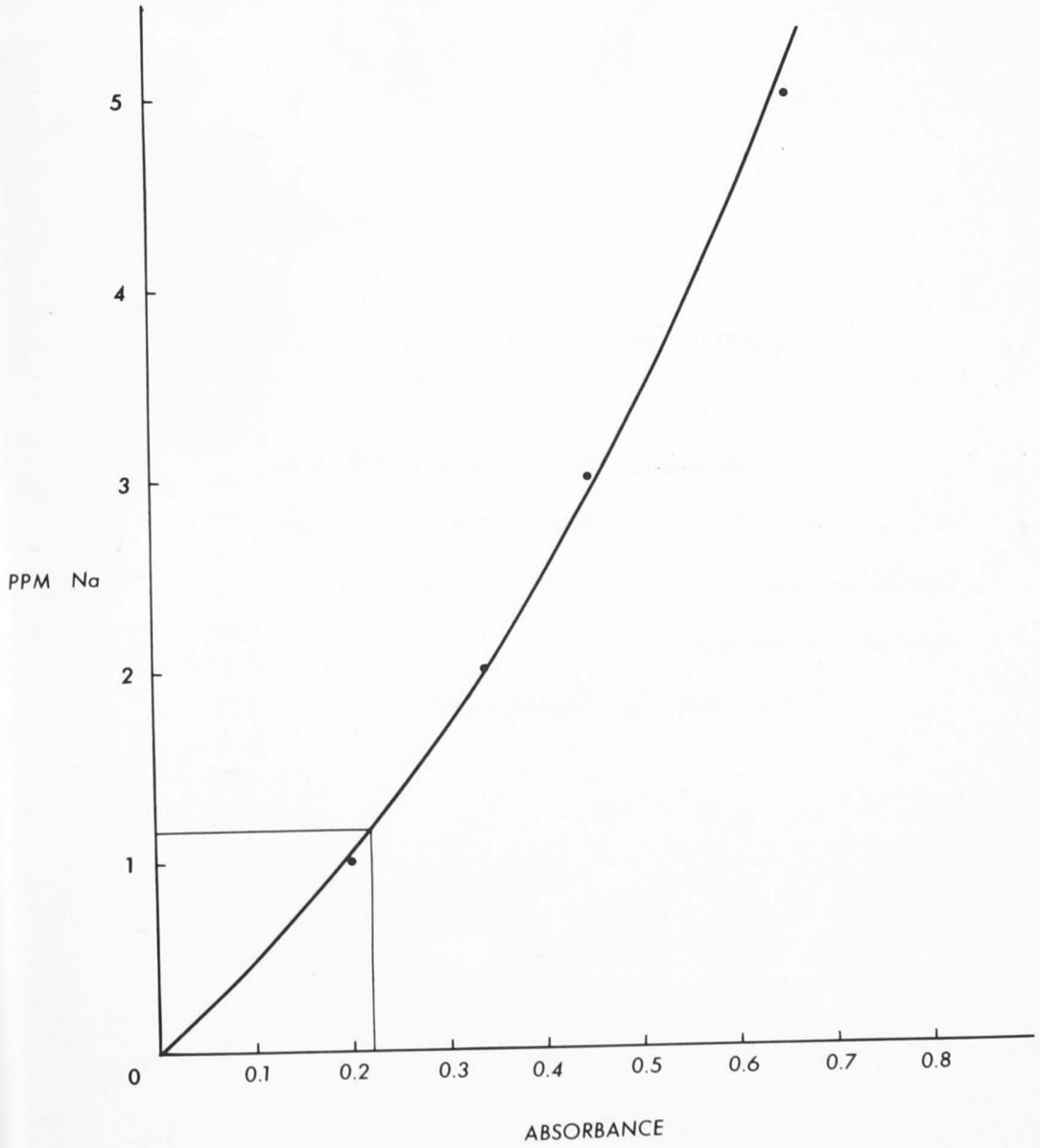
pseudocoel water and similarly for K.

APPENDIX FIGURE 3.1.

Calibration curve of concentration of Na (parts per million) against optical density (or absorbance) for the Atomic Absorption Spectrophotometer.

(Line drawn by inspection).

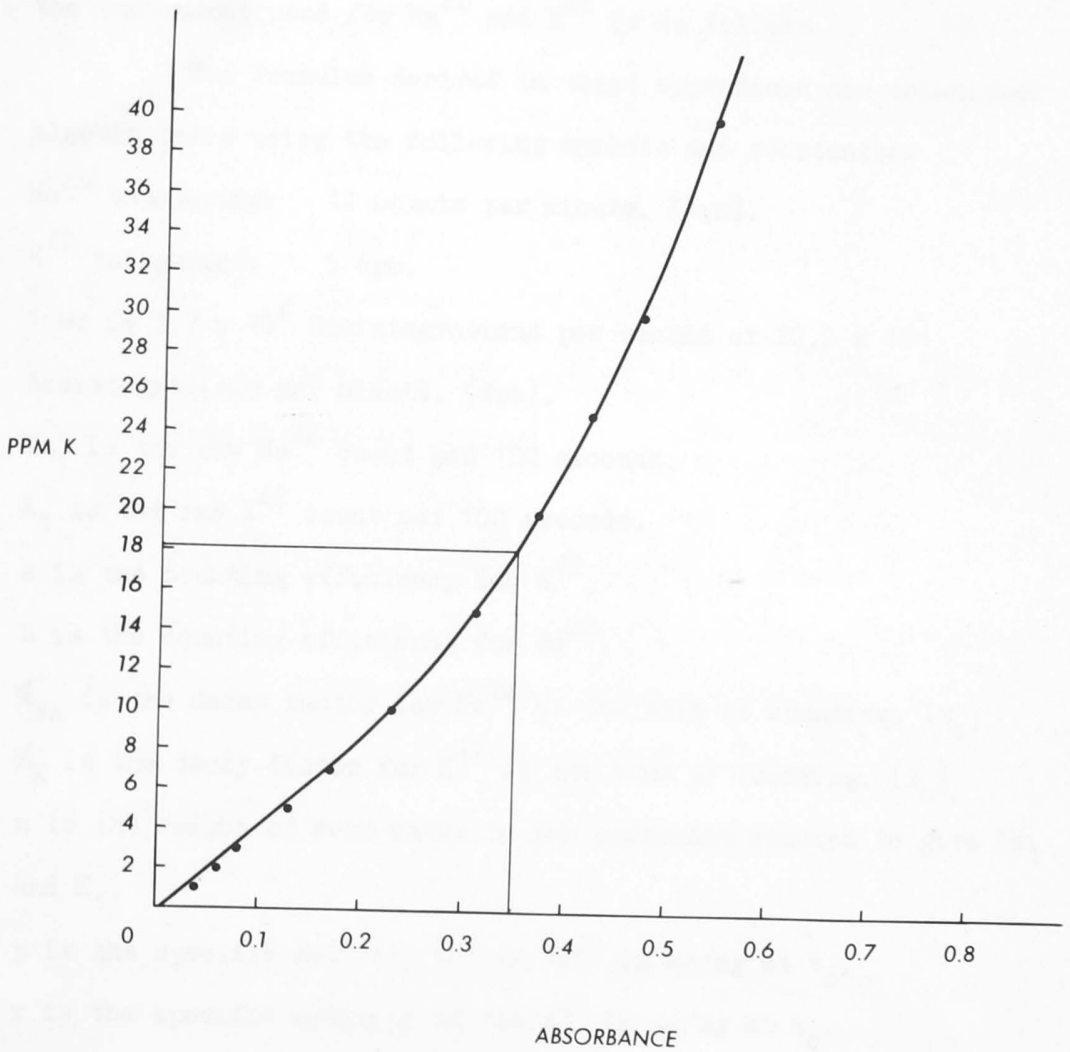
PPM Na


5
4
3
PPM Na
2
1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
ABSORBANCE

APPENDIX FIGURE 3.2.

Calibration curve of concentration of K (parts per million) against optical density (or absorbance) for the Atomic Absorption Spectrophotometer.

(Line drawn by inspection).


PPM K
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
ABSORBANCE

APPENDIX 4.1.

The method used to calculate the counting efficiency of the instrument used for Na^{24} and K^{42} is as follows.

The formulae derived in these appendices are calculated algebraically using the following symbols and constants:-

Na^{24} background: 12 counts per minute. (cpm).

K^{42} background: 5 cpm.

1 μc is 3.7×10^4 disintegrations per second or 22.2×10^5 disintegrations per minute. (dpm).

Na_1 is the raw Na^{24} count per 100 seconds.

K_1 is the raw K^{42} count per 100 seconds.

a is the counting efficiency for K^{42}.

b is the counting efficiency for Na^{24}.

$\%_{Na}$ is the decay factor for Na^{24} at the time of counting. (t_c)

$\%_K$ is the decay factor for K^{42} at the time of counting. (t_c)

n is the weight of worm water in the component counted to give Na_1 and K_1.

p is the specific activity of the Na^{24} in μc/mg at t_o.

r is the specific activity of the K^{42} in μc/mg at t_o.

v is the volume of Na^{24} diluted stock added to the culture.

u is the volume of K^{42} diluted stock added to the culture.

s is the specific activity of the Na^{24} stock in μc/ml.

t is the specific activity of the K^{42} stock in μc/ml.

d is the dilution factor of the original Na^{24} stock.

e is the dilution factor of the original K^{42} stock.

f is the volume of the culture.

$\%_{Na_1}$ is the decay factor for Na^{24} at the time of counting K^{42}.

$\%_{K_1}$ is the decay factor for K^{42} at the time of counting Na^{24}.

t_o is the zero time. (1600 hours on the day of delivery).

t_c is the time of counting.

Na_c is the Na^{24} contamination in the K^{42} count.

K_c is the K^{42} contamination in the Na^{24} count.

To estimate the efficiency of counting Na^{24} and K^{42} the following formula was used.

For Na^{24}:

1. Estimate the real count of the solution as follows:

v μl of Na^{24} stock of specific activity s μc/ml at t_o was added to the medium. The dilution factor of the stock was d.

The specific activity of the stock was s μc/ml.

i.e. s μc in 10^3 μl.

Diluted by factor d, $\therefore \frac{s}{d}$ μc in 10^3 μl.

v μl were added $\therefore$ y μc were added where $y = \frac{v \times s}{d \times 10^3}$ μc,

which produces $\frac{v \times s \times 22.2 \times 10^5}{d \times 10^3}$ dpm.

$\therefore$ the true count at t_o is: $\frac{v \times s \times 22.2 \times 10^2}{d}$ dpm.

From initial cross talk counts, i.e. the Na^{24} count on the K^{42} channel, and vice versa, the contamination of Na^{24} in K^{42} is 7.2%

of the Na^{24} present at original activity.

$\therefore Na_c$ is 7.2% of the true count.

$$\therefore Na_c = \frac{7.2}{100} \left(\frac{v \times s \times 22.2 \times 10^2}{d} \right) \text{ cpm.}$$

$$= \frac{159.84 \times v \times s}{d} \text{ cpm.}$$

Thus at the time of counting K^{42}, Na^{24} contamination is

$$Na_c = \frac{159.84 \times v \times s \times \%Na_1}{d \times 10^2}$$

Before Na^{24} counting efficiency can be estimated the contamination or cross talk factor of K^{42} in the Na^{24} count must be estimated in the same way. Thus calculate the ideal count of adding u μl of K^{42} stock of specific activity t μc/ml at t_o and diluted by factor e.

$\therefore$ there are t μc/ml or 10^3 μl,

diluted by factor e = $\frac{t}{e}$ μc / 10^3 μl,

Thus added y μc in u μl, where y = $\frac{u \times t}{10^3 \times e}$ μc.

which produces $\frac{u \times t \times 22.2 \times 10^5}{e \times 10^3}$ dpm.

$\therefore$ true K^{42} count is $\frac{u \times t \times 22.2 \times 10^2}{e}$ dpm.

K^{42} contamination in the Na^{24} count is 1.9% of the K^{42} activity present.

$$\therefore K_c = \frac{1.9}{100} \times \frac{u \times t \times 22.2 \times 10^2}{e} \text{ dpm.}$$

$$= \frac{42.18 \times u \times t}{e} \text{ dpm.}$$

Thus at the time of counting Na^{24}, K^{42} contamination is:

$$\frac{42.18 \times u \times t \times \%_{K_1}}{e \times 10^2} \text{ dpm.}$$

Thus the K^{42} contamination in the Na^{24} count is known, and can be deducted from the Na^{24} count (Na_1), and vice versa.

Calculation of the counting efficiency involves a series of steps which are:-

1. Correct Na_1 (count per 100 seconds) to cpm and deduct the background count per minute. (12 cpm).
2. Correct the count obtained on 0.2 mls to the volume of the culture, (f mls), to which the original isotope additions were made.
3. Deduct the K^{42} contamination, (K_c).
4. Correct for isotope decay.
5. Calculate the counting efficiency, (b%).

1. Correct for background and convert to cpm.

   Na_1 is the raw count,

$$Na_2 = \frac{Na_1 \times 60}{100} - 12 \text{ cpm.}$$

$$\therefore \quad Na_2 = \frac{Na_1 \times 6}{10} - 12 \text{ cpm.}$$

2. Correct for volume, (0.2 mls to f mls.)

$$Na_3 = \frac{Na_2 \times f}{0.2} \text{ cpm}$$

$$\therefore \quad Na_3 = \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{f}{0.2} \text{ cpm.}$$

3. Deduct K^{42} contamination:

$$Na_4 = Na_3 - K_c,$$

$$\therefore \quad Na_4 = \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{f}{0.2} - \frac{42.18 \times u \times t \times \%K_1}{100 \times e} \text{ cpm.}$$

4. Correct for Na^{24} decay: (t_o to t_c):

$$Na_5 = \frac{Na_4 \times 100}{\%Na}$$

$$\therefore \quad Na_5 = \left[\left(\frac{Na_1 \times 6}{10} - 12\right) \frac{f}{0.2} - \frac{42.18 \times u \times t \times \%K_1}{100 \times e}\right] \frac{100}{\%Na} \text{ cpm}$$

5. Estimate counting efficiency for Na^{24}.

$$\text{Efficiency } b\% = \frac{Na_5 \times 100}{\text{true count}} \%$$

$$= \frac{Na_5 \times 100}{\frac{v \times s \times 22.2 \times 10^2}{d}} \%$$

$$= Na_5 \times 100 \times \frac{d}{v \times s \times 22.2 \times 10^2} \%$$

$$= \left[\left(\frac{Na_1 \times 6}{10} - 12\right) \frac{f}{0.2} - \frac{42.18 \times u \times t \times \%K_1}{100 \times e}\right] \frac{100 \times d \times 100}{\%Na \times v \times s \times 22.2 \times 10^2} \%$$

$$\therefore \quad b\% = \left[\left(\frac{Na_1 \times 6}{10} - 12\right) \frac{f}{0.2} - \frac{42.18 \times u \times t \times \%K_1}{100 \times e}\right] \frac{100 \times d}{\%Na \times v \times s \times 22.2} \%$$

Where $b\%$ is the counting efficiency for Na^{24}.

The counting efficiency, a%, for K^{42} is derived in the same way and gives the result:

$$\left[\left(\frac{K_1 \times 6}{10} - 5\right)\frac{f}{0.2} - \frac{159.84 \times v \times s \times \%_{Na_1}}{100 \times d}\right]\frac{100 \times e}{\%_K \times u \times t \times 22.2}\ \%$$

This efficiency then requires correction for the 82% β^- abundance of K^{42} decay.

$$\text{i.e. } a\% = \left[\left(\frac{K_1 \times 6}{10} - 5\right)\frac{f}{0.2} - \frac{159.84 \times v \times s \times \%_{Na_1}}{100 \times d}\right]\frac{10^4 \times e}{\%_K \times u \times t \times 22.2 \times 82}\ \%$$

APPENDIX 4.2.

The Na^{24} and K^{42} content of the media and worm components can be estimated once the counting efficiency has been established.

To estimate the Na^{24} in the medium the following steps are involved:

1. Correct count per 100 seconds to cpm and deduct the background.
2. Correct for counting efficiency.
3. Deduct K^{42} contamination.
4. Correct for isotope decay to time t_o.
5. Calculate the content of the count in μc. i.e. $\mu c/0.2$ mls.
6. Calculate the content per 1 ml.
7. Calculate the content in mg/ml.
8. Convert to μM/ml.

1. Correct to cpm and deduct background:

$$Na_2 = \frac{Na_1 \times 6}{10} - 12 \text{ cpm.}$$

2. Correct for counting efficiency:

$$Na_3 = \frac{Na_2 \times 100}{b} \text{ cpm.}$$

$$\therefore Na_3 = \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{100}{b} \text{ cpm.}$$

3. Deduct K_c, where $K_c = \left(\frac{K_1 \times 6}{10} - 5\right) \frac{10^2}{41 \times a}$ cpm.

This formula is derived as follows:

Take the raw count per 100 seconds of K^{42} on its own channel as K_1 at a% counting efficiency.

Correct to cpm and deduct background.

$$K_2 = \frac{K_1 \times 6}{10} - 5 \text{ cpm}$$

Correct for efficiency a%

$$K_3 = K_2 \times \frac{100}{a} \text{ cpm},$$

$$= \left(\frac{K_1 \times 6}{10} - 5\right) \frac{100}{a} \text{ cpm.}$$

Correct for abundance, (82%)

$$K_4 = K_3 \times \frac{100}{82}$$

$$= \left(\frac{K_1 \times 6}{10} - 5\right) \frac{100 \times 100}{82 \times a} \text{ cpm.}$$

This count is now equivalent to the true count of the sample given by: $\frac{u \times t \times 22.2 \times 10^2}{e}$ cpm.

and 2% of this is counted on the Na channel. $\therefore K^{42}$ contamination is given by:

$$K_c = \frac{K_4 \times 2}{100} \text{ cpm.}$$

$$= \left(\frac{K_1 \times 6}{10} - 5\right) \frac{10^4 \times 2}{10^2 \times 82 \times a} \text{ cpm}$$

$$= \left(\frac{K_1 \times 6}{10} - 5\right) \frac{10^2}{41 \times a} \text{ cpm.}$$

and $Na_4 = Na_3 - K_c$

$$\therefore Na_4 = \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{100}{b} - \left(\frac{K_1 \times 6}{10} - 5\right) \frac{100}{41 \times a} \text{ cpm.}$$

4. Correct for isotope decay at the time of counting, t_c.

$$Na_5 \text{ at } t_o = \frac{Na_4 \times 100}{\%_{Na}} \text{ cpm.}$$

$$= \left[\left(\frac{Na_1 \times 6}{10} - 12\right) \frac{100}{b} - \left(\frac{K_1 \times 6}{10} - 5\right) \frac{100}{41 \times a}\right] \frac{100}{\%_{Na}} \text{ cpm.}$$

5. Calculate the content of this count (0.2 ml) in μc.

$$\text{Na content}_1 = \frac{Na_5}{22.2 \times 10^5} \ \mu c \ / \ 0.2 \text{ ml.}$$

6. Calculate the content in μc / ml. (i.e. x 5)

$$\text{Na content}_2 = \text{Na content}_1 \times 5$$

$$= \frac{Na_5 \times 5}{22.2 \times 10^5} \ \mu c \ / \text{ ml.}$$

7. Calculate the content in mg/ml for specific activity p μc/mg.

$$\text{Na content}_3 = \frac{\text{Na content}_2}{p} \text{ mg/ml.}$$

$$= \frac{Na_5 \times 5}{22.2 \times 10^5 \times p} \text{ mg/ml.}$$

8. Estimate the concentration in $\mu M/ml$ (molecular weight of Na is 23).

$$\text{Na content}_4 = \frac{\text{Na content}_3}{23} \ \mu M/ml.$$

$$= \frac{Na_5 \times 5 \times 10^3}{22.2 \times 10^5 \times p \times 23} \ \mu M/ml.$$

$\therefore$ the culture contains $\dfrac{Na_5 \times 5 \times 10^3}{22.2 \times 10^5 \times p \times 23} \ \mu M/ml.$

$$= \left[\left(\frac{Na_1 \times 6}{10} - 12\right)\frac{100}{b} - \left(\frac{K_1 \times 6}{10} - 5\right)\frac{100}{41 \times a}\right] \frac{100 \times 5 \times 10^3}{\%_{Na} \times 22.2 \times 10^5 \times 23 \times p}$$

$\therefore \mu M/ml \ Na^{24}$ is given by:

$$\left[\left(\frac{Na_1 \times 6}{10} - 12\right)\frac{100}{b} - \left(\frac{K_1 \times 6}{10} - 5\right)\frac{100}{41 \times a}\right] \frac{0.009792}{\%_{Na} \times p}$$

To estimate the K^{42} content of each culture the same procedure was used.

Estimate the Na^{24} contamination:

The raw count of Na^{24} on its own channel is Na_1 per 100 seconds at efficiency b%. Estimate cpm and deduct background:

$$Na_2 = \frac{Na_1 \times 6}{10} - 12 \ \text{cpm.}$$

Correct for efficiency:

$$Na_3 = \frac{Na_2 \times 100}{b} \ \text{cpm.}$$

$$\therefore \ Na_3 = \left(\frac{Na_1 \times 6}{10} - 12\right)\frac{100}{b} \ \text{cpm.}$$

This is now equivalent to the true count given by:

$$\frac{v \times s \times 22.2 \times 10^2}{d} \text{ cpm.}$$

and contamination is 7.2% of the true Na^{24} count.

$$\therefore Na_c = \frac{Na_3 \times 7.2}{100} \text{ cpm.}$$

$$= \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{100}{b} \times \frac{7.2}{100}$$

$$= \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{7.2}{b} \text{ cpm.}$$

To estimate the K^{42} content of the medium with counting efficiency a%:

Na_1 and K_1 are the counts per 100 seconds on 0.2 ml aliquots of medium.

1. Correct to cpm and deduct background.

$$K_2 = \frac{K_1 \times 6}{10} - 5 \text{ cpm.}$$

2. Correct for counting efficiency:

$$K_3 = \frac{K_2 \times 100}{a}$$

$$K_3 = \left(\frac{K_2 \times 6}{10} - 5\right) \frac{100}{a} \text{ cpm.}$$

3. Correct K_3 for abundance:

$$K_4 = \left(\frac{K_3 \times 100}{82}\right)$$

$$= \left(\frac{K_1 \times 6}{10} - 5\right) \frac{100 \times 100}{82 \times a}$$

$$= \left(\frac{K_1 \times 6}{10} - 5\right) \frac{10^4}{82 \times a} \text{ cpm.}$$

4. Deduct the Na^{24} contamination.

$$K_5 = K_4 - Na_c$$

$$= \left(\frac{K_1 \times 6}{10} - 5\right) \frac{10^4}{82 \times a} - \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{7.2}{b} \text{ cpm.}$$

5. Correct for isotope decay:

$$K_6 = \frac{K_5 \times 100}{\%_K}$$

$$= \left[\left(\frac{K_1 \times 6}{10} - 5\right) \frac{10^4}{82 \times a} - \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{7.2}{b}\right] \frac{100}{\%\,K} \text{ cpm.}$$

6. Estimate the content of the count in μc.

$$K \text{ content}_1 = \frac{K_6}{22.2 \times 10^5} \ \mu c.$$

7. Estimate the content in the count in $\mu c/ml$ from the 0.2 ml count. (i.e. x 5)

$$K \text{ content}_2 = K \text{ content}_1 \times 5.$$

$$= \frac{K_6 \times 5}{22.2 \times 10^5} \ \mu c/ml$$

8. Estimate the culture K^{42} content in mg/ml from the specific activity r $\mu c/mg$.

$$K \text{ content}_3 = \frac{K \text{ content}_2}{r}$$

$$= \frac{K \text{ content}_2 \times 5}{22.2 \times 10^5 \times r} \text{ mg/ml.}$$

9. Estimate the concentration in μm/ml. The molecular weight of K is 39.

$$K\ \text{content}_4 = \frac{K\ \text{content}_3}{39}$$

$$= \frac{K_6 \times 5 \times 10^3}{22.2 \times 10^5 \times r \times 39}$$

Thus the concentration of K in μM/ml is:

$$\left[\left(\frac{K_1 \times 6}{10} - 5\right)\frac{10^4}{82xa} - \left(\frac{Na_1 \times 6}{10} - 12\right)\frac{7.2}{b}\right]\frac{100}{\%_K} \times \frac{5 \times 10^3}{22.2 \times 10^5 \times r \times 39}$$

$$= \left[\left(\frac{K_1 \times 6}{10} - 5\right)\frac{10^4}{82xa} - \left(\frac{Na_1 \times 6}{10} - 12\right)\frac{7.2}{b}\right]\frac{0.005775}{\%_K \times r}\ \mu M/ml.$$

APPENDIX 4.3.

To estimate the Na^{24} and K^{42} content of the worm components.

Na^{24} content in $\mu M/ml$, is given by the following method:

The K^{42} contamination is given by: (see appendix 4.2.)

$$K_c = \left(\frac{K_1 \times 6}{10} - 5\right) \frac{100}{41 \times a}$$

where K_1 is the K^{42} count of the component on its own channel per 100 seconds at efficiency a%.

Take the raw count of Na^{24} on its own channel as Na_1 per 100 seconds at efficiency b%.

1. Correct to cpm and deduct background.

$$Na_2 = \frac{Na_1 \times 6}{10} - 12 \text{ cpm.}$$

2. Correct for counting efficiency b%.

$$Na_3 = \frac{Na_2 \times 100}{b} \text{ cpm.}$$

$$= \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{100}{b} \text{ cpm.}$$

3. Deduct K^{42} contamination:

$$Na_4 = Na_3 - K_c.$$

$$= \left(\frac{Na_1 \times 6}{10} - 12\right) \frac{100}{b} - \left(\frac{K_1 \times 6}{10} - 5\right) \frac{100}{41 \times a} \text{ cpm.}$$

4. Correct for isotope decay.

$$Na_5 = \frac{Na_4 \times 100}{\%Na}$$

$$= \left[\left(\frac{Na_1 \times 6}{10} - 12\right)\frac{100}{b} - \left(\frac{K_1 \times 6}{10} - 5\right)\frac{100}{41 \times a}\right]\frac{100}{\%Na} \text{ cpm.}$$

5. Calculate the content of the count in μc.

$$\text{Na content}_1 = \frac{Na_5}{22.2 \times 10^5} \mu c.$$

6. Correct volume from 0.2 ml to total extract volume 0.5 ml.

$$\text{Na content}_2 = \frac{\text{Na content}_1 \times 0.5}{0.2}$$

$$\text{Na content}_2 = \frac{2.5 \times Na_5}{22.2 \times 10^5} \mu c/0.5 \text{ ml.}$$

Thus the Na content$_2$ is the total Na^{24} content of the worm components as they were redissolved in 0.5 ml. The water weight of this component was n gms. Thus this Na^{24} was dissolved in n gms of water.

Calculate the content/ml from:

7. $$\text{Na content}_3 = \frac{\text{Na content}_2}{n} \mu c/n \text{ gms of water.}$$

$$= \frac{Na_5 \times 2.5}{22.2 \times 10^5 \times n} \mu c/ml.$$

8. Calculate this content as mg/ml from the specific activity p μc/mg.

$$\text{Na content}_4 = \frac{\text{Na content}_3}{p} \text{ mg/ml.}$$

$$\therefore \text{Na content}_4 = \frac{Na_5 \times 2.5}{22.2 \times 10^5 \times n \times p} \text{ mg/ml.}$$

9. Estimate this content as μM/ml.

$$\text{Na content}_5 = \frac{\text{Na content}_4}{23}$$

$$= \frac{Na_5 \times 2.5 \times 10^3}{22.2 \times 10^5 \times n \times p \times 23} \text{ } \mu\text{M/ml.}$$

$$= \frac{Na_5 \times 0.000049}{n \times p} \text{ } \mu\text{M/ml.}$$

Thus Na content in μM/ml is:

$$\left[\left(\frac{Na_1 \times 6}{10} - 12\right)\frac{100}{b} - \left(\frac{K_1 \times 6}{10} - 5\right)\frac{100}{41 \times a}\right]\frac{100 \times 0.000049}{\%_{Na} \times n \times p} \text{ } \mu\text{M/ml.}$$

K content may be calculated in the same way with an abundance correction. The result is: K content μM/ml:

$$= \left[\left(\frac{K_1 \times 6}{10} - 5\right)\frac{10^4}{82 \times a} - \left(\frac{Na_1 \times 6}{10} - 12\right)\frac{7.2}{b}\right]\frac{0.002888}{\%_K \times n \times r} \text{ } \mu\text{M/ml.}$$

The results calculated by these formulae have been corrected for recovery efficiency by multiplying by the factors 1.1250 for Na^{24} and 1.2686 for K^{42}.

The concentration of Na^{24} and K^{42} in the medium and the worm components is thus known in μM/ml. The concentration factor may be calculated from:

$$\text{concentration factor} = \frac{\text{concentration in the worm } (\mu\text{M/ml})}{\text{concentration in the medium } (\mu\text{M/ml})}$$

APPENDIX 4.4.

In one of the experiments described in chapter 4 the loss of isotope from the worms was calculated. The amount lost was calculated as μM Na or μM K/ml in the worm and as the per cent loss. y is the concentration (μM/ml) recorded in f mls of culture after incubating a labelled worm. n is the weight of water in the worm.

$\therefore$ y μM/ml recorded in f mls,

total content = f x y μM.

$\therefore$ μM/ml worm water = $\frac{f \times y}{n}$

$\therefore$ μM/ml worm water lost in the second culture (L) is

$$L = \frac{f \times y}{n} \quad \mu M/ml.$$

The total initial concentration, (I) = concentration still in the worm + $\frac{f \times y}{n}$ μM/ml.

$$\therefore \% \text{ loss} = \frac{\mu M/ml \text{ lost (L)}}{\text{total initial conc (I)}} \times \frac{100}{1}$$

$$= \frac{L}{I} \times \frac{100}{1}$$

APPENDIX 5.1.

The tritium oxide content of M. dubius or one of its components was calculated as follows:

Let the sample count be y cpm at counting efficiency E%, with background B cpm. The specific activity of the tritium oxide was 20 μc/ml. Let the water weight of the worm or component be n grams.

1. Raw count: y cpm / n g worm water.

2. Correct background: = y - B cpm.

3. Correct to 100% counting efficiency:

$$= \frac{(y - B) \times 100}{E\%} \text{ cpm / n g worm water.}$$

4. Convert to μc: $$= \frac{(y - B) \times 100}{E\% \times 22.2 \times 10^5} \mu\text{c / n g worm water.}$$

5. Convert to ml: $$= \frac{(y - B) \times 100}{E\% \times 22.2 \times 10^5 \times 20} \text{ml / ng worm water.}$$

6. Convert to μls: $$= \frac{(y - B) \times 100 \times 10^3}{E\% \times 22.2 \times 10^5 \times 20} \mu\text{l / n g worm water.}$$

7. Estimate as μl/g worm water:

$$= \frac{(y - B) \times 10^5}{E\% \times 22.2 \times 10^5 \times 20 \times n} \mu\text{l/ml worm water.}$$

Wang, Robinson and Edelman (1953) estimated that tritium oxide diffused at a rate 14% slower than $H_2^1O^{18}$ which they consider to be the best tracer of water movement. Thus all the tritium content figures have been corrected x $\frac{100}{86}$.

8. Correct for diffusion rates:

$$= \frac{(y - B) \times 10^5 \times 10^2}{E\% \times 22.2 \times 10^5 \times 20 \times n \times 86} \ \mu l/ml \text{ worm water.}$$

$$= \frac{(y - B) \times 10^7}{E\% \times 44.4 \times 10^6 \times n \times 86} \ \mu l/ml \text{ worm water.}$$

$$= \frac{(y - B) \times 10}{E\% \times 3818.4 \times n} \ \mu l/ml \text{ worm water.}$$

The tritium oxide content of the medium in μl H_2^3O/ml of medium was calculated from 0.5 ml counts as follows:

1. Count at E% efficiency is: y cpm/0.5 ml.
2. Deduct background: = y - B cpm/0.5 ml.
3. Correct to 100% counting efficiency:

$$= \frac{(y - B) \times 100}{E\%} \text{ cpm/0.5 ml.}$$

4. Convert to μc. $= \frac{(y - B) \times 100}{E\% \times 22.2 \times 10^5} \ \mu c/0.5$ ml.

5. Convert to μl. $= \frac{(y - B) \times 100 \times 10^3}{E\% \times 22.2 \times 10^5 \times 20} \ \mu l/0.5$ ml.

6. Convert to μl/ml. $= \frac{(y - B) \times 10^5 \times 2}{E\% \times 22.2 \times 10^6 \times 2} \ \mu l/ml.$

$$= \frac{(y - B)}{E\% \times 222} \ \mu l/ml.$$

To estimate the total water movement the movement of H_2^3O is multiplied by the ratio of H_2^1O to H_2^3O = R.

There are $\frac{(y - B)}{E\% \times 222}$ μl H_2^3O per 10^3 μl medium.

$\therefore$ there is 1 μl H^3_2O per R μl of culture.

$$\therefore R = \frac{10^3 \times 222 \times E\%}{(y - B)}$$

The counting efficiency was determined by internal standardisation of each sample. i.e. a 50 μl aliquot of diluted H^3_2O stock, which produced 11,100 dpm, was added to each sample. after it had been counted for the H^3 extracted from the worm, or contained in the medium. This is count C_1. The sample was recounted after internal standardisation, giving count C_2. The counting efficiency is given by:

$$E\% = \frac{C_2 - C_1}{11,100} \times \frac{100}{1}$$

The mean medium and worm component counting efficiency was estimated and used throughout the experiment.

APPENDIX 5.2.

The following procedure was used to estimate the permeability coefficient of the tegument of M. dubius. The quantity of water which had penetrated the worm in the 10 minute culture period was estimated from the calculated content in μl/g worm water by multiplying the individual estimates by the water weight of that worm. The weight of the whole worm, W gms, was used to estimate the surface area of the worms individually by the formulae:

Surface area, females (cm^2) = body weight $^{2/3}$ x 19.07.

Surface area, males (cm^2) = body weight $^{2/3}$ x 15.32.

The rate constant μl/worm/10 minutes was estimated as ml/worm/second, and then as ml/cm^2/second from the above formulae.

The osmotic pressure was taken as the figures obtained from the freezing point depression estimates of chapter 3. The osmotic pressure difference used was the difference between Hanks balanced salt solution and the worm, as real osmotic pressures, in atmospheres, after adjustment for temperature. The real osmotic pressures of the worms at the various temperatures is shown in appendix table 5.2.1.

APPENDIX TABLE 5.2.1. The real osmotic pressures of male and female M. dubius and Hanks balanced salt solution at 310, 303, 293 and 283 ^{o}K in mm Hg.

	Temperature (^{o}K)			
Item	310	303	293	283
Female	11,015	10,766	10,411	10,056
Male	15,300	14,954	14,460	13,967
Hanks	5,740	5,610	5,425	5,240

The differences in real osmotic pressure are shown in appendix table 5.2.2. in mm Hg and atmospheres.

APPENDIX TABLE 5.2.2. The difference in real osmotic pressure between male and female M. dubius and Hanks balanced salt solution in mm Hg(and atmospheres) at various temperatures.

	Temperature (^{o}K)			
Worm	310	303	293	283
Female	5275 (6.94)	5156 (6.78)	4986 (6.56)	4816 (6.33)
Male	9560 (12.57)	9344 (12.29)	9035 (11.88)	8727 (11.48)

The estimate as ml/cm^2/second was then divided by the real osmotic pressure difference to give the permeability constant in ml/cm^2/second/atmosphere.

APPENDIX 5.3.

To determine the percentage loss of water from labelled worms into Hanks balanced salt solution the following procedure was used:

The H_2^3O content of the worm after the second incubation was estimated by the method shown in appendix 5.1. i.e. as μl/g worm water (A). The H_2^3O content of the medium was estimated by the method shown in appendix 5.1. as μl/ml culture (B). The culture volume was 5.0 mls. Therefore the loss was 5 x B μls. This loss was estimated as μl/g worm water by dividing by the weight of worm water (n gram). i.e.

$$\mu\text{l/g worm water lost} = \frac{5 \times B}{n} \quad (C).$$

Thus the original total worm content was:

$$A + \frac{5 \times B}{n}$$

or $A + C$. μl/g worm water.

Thus the percentage loss is given by:

$$\frac{C}{A + C} \times \frac{100}{1} \%.$$

APPENDIX 6.1.

The C^{14} labelled nutrient content of worms and extract media counted with a Geiger-Müller Tube was calculated in the following way:

The Geiger-Müller Tube was standardised with a C^{14} impregnated disc which produced 12,762 dpm. The background was estimated from a clean planchet. The efficiency of the tube was estimated to be 13.1% with a background count of 8 cpm. All counts were made over 300 seconds.

All test counts were made on 50 μl aliquots dried on flat aluminium planchets. Sample geometry was controlled by redissolving the sample on the planchet with 0.2 ml of 90% ethanol and redrying the planchet.

If the test count is y cpm with efficiency E% the content of the medium or the worm extract is given by the following steps:

1. Raw count is y cpm.
2. Correct for background: $y - 8$ cpm.
3. Correct for efficiency: $\frac{(y - 8) \times 100}{E\%}$ cpm.

This count is taken from a 50 μl aliquot of worm extract or medium. Thus to estimate the total count, multiply by the volume of the solution ($a \times 10^3$ μl).

4. Correct for volume: $\frac{(y - 8) \times 100 \times a \times 10^3}{E\% \times 50}$ cpm/a mls

5. Convert to μc: $\dfrac{(y - 8) \times 100 \times a \times 10^3}{E\% \times 50 \times 22.2 \times 10^5}$ $\mu c/a$ mls.

6. Convert to μM of nutrient with specific activity S $\mu c/\mu M$.

$$= \frac{(y - 8) \times a}{E\% \times 50 \times 22.2 \times S} \quad \mu M/a \text{ mls.}$$

$$= \frac{(y - 8) \times a}{E\% \times S \times 11.1 \times 10^2} \quad \mu M/a \text{ mls.}$$

The formula gives the μM of nutrient in the total culture (a = 6.5 mls) or worm extract solution (a = 3.0 mls). Thus to estimate the concentration of the medium in μM/ml divide by a = 6.5.

$\therefore$ medium concentration = $\dfrac{(y - 8)}{E\% \times S \times 11.1 \times 10^2}$ $\mu M/ml$.

To estimate the amino acid concentration within the worm, divide by the weight of water in the worm component analysed, n grams, as this volume originally contained the labelled contents of the 3.0 mls of worm extract.

$\therefore$ μM/ml worm water = $\dfrac{(y - 8) \times a}{E\% \times S \times 11.1 \times 10^2 \times n}$ μM/ml worm water.

The counting efficiency is 13.1% so the formulae become:

medium concentration = $\dfrac{(y - 8)}{13.1 \times 11.1 \times 10^2 \times S}$ $\mu M/ml$.

$$= \frac{(y - 8)}{14{,}541 \times S} \quad \mu M/ml.$$

and the worm concentration is given by:

$$= \frac{(y - 8) \times 3}{13.1\% \times 11.1 \times 10^2 \times S \times n}$$

$$= \frac{(y - 8) \times 3}{14{,}541 \times S \times n} \quad \mu M/ml \text{ worm water.}$$

$$= \frac{(y - 8)}{4,847 \times S \times n} \quad \mu M/ml \text{ worm water.}$$

The specific activities of the materials used were:

Leucine: 6.6 $\mu c/\mu M$.

Alanine: 20.2 $\mu c/\mu M$.

Serine: 7.42 $\mu c/\mu M$.

Glutamic acid: 130.0 $\mu c/\mu M$.

Sucrose: 11.8 $\mu c/\mu M$.

Chlorella protein: 5.0 $\mu c/\mu M$.*

Chlorella protein hydrolysate: 8.5 $\mu c/\mu M$.*

* Estimated from the weighted average molecular weight of the amino acids and the specific activity of the material in $\mu c/mg$, 200 $\mu c/mg$ for the protein and the protein hydrolysate.

Thus the formulae used were:

Compound	Medium concentration ($\mu M/ml$)	Worm concentration ($\mu M/ml$)
Leucine	$\frac{(y - 8)}{95,970.6}$	$\frac{(y - 8)}{31.990.2 \times n}$
Alanine	$\frac{(y - 8)}{293,738.2}$	$\frac{(y - 8)}{97,909.4 \times n}$
Serine	$\frac{(y - 8)}{107,894.2}$	$\frac{(y - 8)}{35,964.7 \times n}$
Glutamic acid	$\frac{(y - 8)}{1,890,330.0}$	$\frac{(y - 8)}{630,110.0 \times n}$
Sucrose	$\frac{(y - 8)}{171,583.8}$	$\frac{(y - 8)}{57,194.6 \times n}$
Chlorella protein	$\frac{(y - 8)}{72,705.0}$	$\frac{(y - 8)}{24,235.0 \times n}$
Chlorella protein hydrolysate	$\frac{(y - 8)}{123,598.5}$	$\frac{(y - 8)}{41,199.5 \times n}$

APPENDIX 6.2.

The worm C^{14} - U - L - leucine content was determined with the liquid scintillation counter and the concentration calculated by the following method:

y_n is the individual count of the three ethanol extracts, at counting efficiency E% and background B cpm. The specific activity of the leucine used was 6.6 $\mu c/\mu M$.

Using the count of the first extract, y_1 estimate the content of leucine in the sample:

1. Raw count is y_1 cpm.
2. Deduct background, $= y_1 - B$ cpm.
3. Correct for counting efficiency:

$$= \frac{(y_1 - B) \times 100}{E\%} \text{ cpm.}$$

4. Convert to μc. $$= \frac{(y_1 - B) \times 100}{E\% \times 22.2 \times 10^5} \mu c.$$

5. Convert to μM. $$= \frac{(y_1 - B) \times 100}{E\% \times 22.2 \times 10^5 \times 6.6} \mu M.$$

There are three ethanol extracts which are all estimated in the same way from counts y_1, y_2 and y_3. Thus the total content is:

6. $$\frac{(y_1 - B) \times 100}{E\% \times 22.2 \times 10^5 \times 6.6} + \frac{(y_2 - B) \times 100}{E\% \times 22.2 \times 10^5 \times 6.6} + \frac{(y_3 - B) \times 100}{E\% \times 22.2 \times 10^5 \times 6.6}$$

$$= \frac{1}{22.2 \times 10^5 \times 6.6} \left\{ \left(\frac{y_1 - B}{E\%}\right) + \left(\frac{y_2 - B}{E\%}\right) + \left(\frac{y_3 - B}{E\%}\right) \right\}$$

If the weight of worm water is n grams the content of the worm component is given by:

$$\frac{1}{22.2 \times 10^5 \times 6.6 \times n} \left\{ \left(\frac{y_1 - B}{E\%}\right) + \left(\frac{y_2 - B}{E\%}\right) + \left(\frac{y_3 - B}{E\%}\right) \right\} \mu M/ml.$$

$$= \frac{1}{146.52 \times 10^5 \times n} \left\{ \left(\frac{y_1 - B}{E\%}\right) + \left(\frac{y_2 - B}{E\%}\right) + \left(\frac{y_3 - B}{E\%}\right) \right\} \mu M/ml.$$

This formula gives the worm content in $\mu m/g$ worm water/min.

The medium content is given by the expression in 5. This is the content per volume of culture counted, a mls. Thus the culture content as $\mu M/ml$ is given by:

$$\frac{(y - B) \times 100}{E\% \times 22.2 \times 10^5 \times 6.6 \times a} \mu M/ml.$$

$$= \frac{(y - B) \times 100}{146.52 \times E\% \times 10^5 \times a} \mu M/ml.$$

$$= \frac{(y - B)}{146.52 \times 10^3 \times E\% \times a} \mu M/ml.$$

The concentration factor is given by:

$$\frac{\mu M/g \text{ worm water}}{\mu M/ml \text{ medium}}$$

Throughout the C^{14} - U - L - leucine experiments in chapters 6 and 7 the counting efficiency was calculated in the following way:

The Beckman CPM 200 Liquid Scintillation Counter is fitted with a Cs^{137} external standard. For the purpose of the

external standard ratio the A and B channels of the counter are preset to detect the 0.51 and 1.18 MeV negatrons of Cs^{137} respectively. The mode of counting is in two parts occupying 6 seconds each. The counts arising from the sample are analysed into two energy bands, i.e. analysed between the A and B channels. The Cs^{137} source is then raised to a position beside the sample bottle and the counts in channels A and B are again accumulated over 6 seconds. The external standard ratio is given by:

$$\frac{B_{2nd\ count} - B_{1st\ count}}{A_{2nd\ count} - A_{1st\ count.}}$$

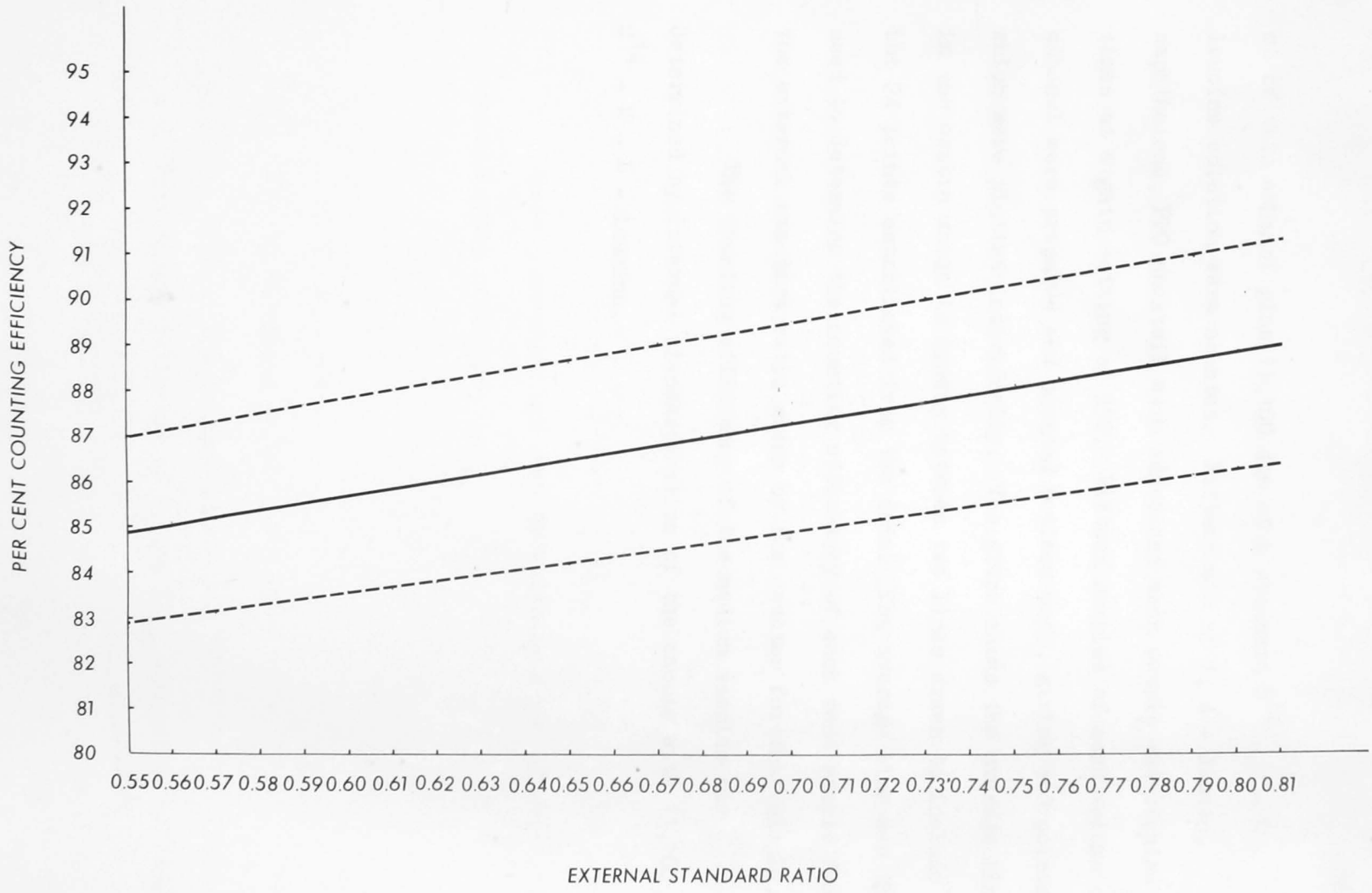
Thus the ratio may vary between zero and infinity.

The external standard ratio is reduced by quenching agents as a greater proportion of the counts fall into the lower energy, A, channel. It may be increased by increasing the gain setting on the instrument. Thus for a particular gain setting with a particular isotope and particular quench, a graph of counting efficiency against external standard ratio may be constructed. The graph shown in appendix figure 6.1 is drawn for C^{14} quenched with 95% ethanol in water at a gain setting of 550.

The results from which this graph was constructed were derived from a series of counts containing varying amounts of 95% ethanol which had been used to extract M. dubius taken from the rat gut. A 100 ml batch of this ethanol was prepared by extracting 50 mixed sex M. dubius individually in 2.0 ml of 95% ethanol, and combining the solutions. Samples containing 0.5, 1.0, 2.0 and 2.5

APPENDIX FIGURE 6.1.

The relationship between external standard ratio and per cent counting efficiency for C^{14} labelled L - leucine extracted from M. dubius in 95% ethanol in water at a gain setting of 550 on a Beckman CPM 200 Liquid Scintillation Counter, using different amounts of quench with a standard concentration of C^{14}.


PER CENT COUNTING EFFICIENCY
95
94
93
92
91
90
89
88
87
86
85
84
83
82
81
80
0.55 0.56 0.57 0.58 0.59 0.60 0.61 0.62 0.63 0.64 0.65 0.66 0.67 0.68 0.69 0.70 0.71 0.72 0.73 0.74 0.75 0.76 0.77 0.78 0.79 0.80 0.81
EXTERNAL STANDARD RATIO

ml of this ethanol plus 11,100 dpm of a standard C^{14} - U - L - leucine solution were counted. Fifteen mls of 1, 4 - dioxan, naphthalene, PPO cocktail were added and each sample was counted 6 times at a gain setting of 550. Fifteen samples of each volume of ethanol were prepared and counted 6 times each, giving 360 points which were plotted individually. The graph shows the average line in the centre which is midway between two lines drawn to include all but 24 points established from the data. The average line was then used to determine the counting efficiency of each test sample from the external standard ratio given by the counter for that sample.

The counting efficiency of the medium samples was determined by internal standardisation of the sample with 11,100 dpm C^{14} - U - L - leucine.

APPENDIX 6.3.

The Michaelis-Menten parameters, K_t and V_{max}, were estimated by the method of Wilkinson (1961). Wilkinson's method was interpreted for the author by Dr. W. Ewens of the Statistics Department, S.G.S., Australian National University. The method given below is that of Dr. Ewens.

The results given in section 6.3.2. provide lists of corresponding substrate concentration (s) in μM/ml and rates of transport (v) in μM/ml worm water/minute. A series of parameters is calculated from these values as follows:-

From s and v calculate

$$w = v^2 \text{ and } y = \frac{v^2}{s}$$

These parameters are used to estimate a set of sums of squares and cross products:-

$$A = \Sigma\, v \times w$$

$$B = \Sigma\, w^2$$

$$C = \Sigma\, v \times y$$

$$D = \Sigma\, w \times y$$

$$E = \Sigma\, y^2$$

Provisional estimates of K_t and V_{max} are estimated from:-

$$K_t^o = \frac{BC - AD}{AE - CD}$$

$$V_{max}^o = \frac{BE - D^2}{AE - CD}$$

The provisional estimates are refined as follows:-

To the provisional estimate of K_t^o add each s value.

A series of values is then estimated from:-

$$f = \frac{V^o \times s}{s + K_t^o}$$

A series of f' values is estimated from:-

$$f' = \frac{- V^o \times s}{(s + K_t^o)^2}$$

A set of sums of squares and cross products is estimated as follows:-

$$A' = \Sigma f^2$$

$$B' = \Sigma (f')^2$$

$$C' = \Sigma f \times f'$$

$$D' = \Sigma v \times f$$

$$E' = \Sigma v \times f'$$

Two correction factors are estimated from this series:-

$$b_1 = \frac{B'D' - C'E'}{A'B' - C'^2}$$

$$b_2 = \frac{A'E' - C'D'}{A'B' - C'^2}$$

The final estimates are given by:-

$$K_t = K_t^o + \frac{b_2}{b_1}$$

$$V_{max} = b_1 \times V_{max}^o$$

The standard error of the estimates is given by:-

Estimate Σv^2 and then

$$s^2 = \frac{\Sigma v^2 - b_1 \times D' - b_2 \times E'}{n - 2}$$

where n is the original number of observations.

The standard error of K_t is given by:-

$$\frac{1}{b_1}\sqrt{\frac{s^2 \times A'}{A'B' - C'^2}}$$

and the standard error of V_{max} is given by:-

$$V^o_{max}\sqrt{\frac{s^2 \times B'}{A'B' - C'^2}}$$

APPENDIX 6.4.

The "t" tests were performed by the method of Li (1964). The mean, and a variance estimate, s^2, were determined for each group of data. The groups of data may be described as w_1, w_2, ----w_n and y_1, y_2, ----y_n, with variance estimates s_w^2 and s_y^2, and means $\bar{w}$ and $\bar{y}$. To test the equality of the variance, and F statistic given by $\frac{s_w^2}{s_y^2}$ was estimated and compared with the value tabulated in the F table at the 5% level of probability for degrees of freedom $n_w - 1$ and $n_y - 1$. If the variance ratio test showed the variances to be non-significantly different a pooled variance was estimated from:-

$$s^2 = \frac{\Sigma w^2 + \Sigma y^2}{n_w - 1 + n_y - 1}$$

where Σw^2 and Σy^2 indicate corrected sums of squares.

"t" is then estimated from:-

$$t = \frac{\bar{w} - \bar{y}}{\sqrt{s^2 (1/n_w + 1/n_y)}}$$

and "t" was compared to the value given in the "t" table at the 5% level of probability and degrees of freedom $n_w + n_y - 2$.

If the variance ratio test showed the variance of the two populations to be significantly different, a "t like statistic", t*, described by Li (1964) was used. The combined variance is estimated from the sum of the variances of the sample means, i.e.

$$s_w^2 / n_w + s_y^2 / n_y$$

The statistic given by Li as t* is given by:-

$$t^* = \frac{\bar{w} - \bar{y}}{\sqrt{\frac{s_w^2}{n_w} + \frac{s_y^2}{n_y}}}$$

The "t" table is entered with degrees of freedom given by:-

$$df^* = \frac{s_w^2/n_w + s_y^2/n_y}{\frac{(s_w^2/n_w)^2}{n_w - 1} + \frac{(s_y^2/n_y)^2}{n_y - 1}}$$

The t* statistic is not as sensitive in detecting significant differences as the "t" test, as the number of degrees of freedom is approximately halved, giving a higher critical "t" value.